[GANN, 52, 265~270; December, 1961]

PRELIMINARY NOTE ON ADENOCARCINOMA OF THE LUNG IN MICE INDUCED WITH 4-NITROQUINOLINE N-OXIDE

(With Plates XXII~XXIV)

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Since the discovery of a spontaneous lung tumor in a mouse by Livingood in 1896, a large number of cases were reported and described in detail by many investigators. And they were variously diagnosed as adenoma, papillary cystadenoma, or adenocarcinoma. However, judging from the description and published illustrations there is but little variation amongst them and they represent a distinct type of epithelial tumor. A few investigators reported that hyperplastic or neoplastic lesions of the lung in mice or in rats have been induced by administration of urethane (1), the carcinogenic hydrocarbons (2, 3, 4, 5), aminofluorene derivatives (6, 7, 8), nitrogen mustard (9), beryllium (10), hydrazid (11), and p-benzoquinone (12, 13) and so on. But there is no well-developed case of an adenocacinoma of the lung in animals induced experimentally.

In the previous paper (14), it has been reported that multiple pulmonary adenomas in mice were induced by a single subcutaneous injection of suitable quantitis of 4-nitroquinoline N-oxide. A further series of experiments has been carried out along this line, and well-established adenocarcinoma of the lung in mice was induced by the repeated injections of 4-nitroquinoline N-oxide. The results are recorded below preliminarily.

Experimental

Twenty female mice of dd strain were used in the experiment. They were 2 to 3 months of age, weighing 15 to 20 g, at the start of the experiment. All the mice were maintained on the mixture of rice and Oriental mouse diet (1 : 1), with an unlimited supply of water. In addition, a bit of green vegetables was given three times a week.

Carcinogen used was 4-nitroquinoline N-oxide which was discovered by Nakahara and his colleagues to be strongly carcinogenic (15, 16). The solvent was a mixture of olive oil and cholesterol (10 : 0.5), and a 0.25 per cent solution of the carcinogen was made up as required.

All the mice were injected subcutaneously with 0.1 ml of the solution of 4-nitroquinoline N-oxide. And the injection was repeated every week for 10 times. Injections were made in rotation in the different subcutaneous sites in the back of the

mice to avoid the possibility of overlapping of the injected spots and thus eliminating the probability of subcutaneous lesions. After 10 injections, having delivered 2.5 mg of 4-nitroquinoline N-oxide, the injection was discontinued, and animals were kept under observation.

Of these, 10 mice survived over 224 days after the first injection of the carcinogen. On the 225 th day, one mouse died with tumor nodules in the lung, and no detectable change in the subcutaneous region. The pulmonary nodules were similar in size and 35 in number. Microscopical examination of the nodules showed them to be papillary cystadenomas, which were identical in structure with those found in the previous work.

On the 234 th day, another mouse died with multiple pearly white nodules in the lung. The number of the tumor nodules was 82 in total. Among them 3 large gray nodules, 3, 4 and 4.5 mm each in diameter, were remarkable in size contrasted to the others (Figs. 1, 2 and 3). And the several deposits of small tumorous nodules were found on the diaphragm, along the chest wall and in the mediastinal region. Lymph nodes lying in the mediastinal region were enlarged. They were proved to be papillary adenocarcinoma in nature upon microscopical examination. All the other mice died later or were killed on the 285 th day, and the result was tabulated.

Table 1. Tumors obtained in the lung of mice treated with subcutaneous injections of 4-nitroquinoline N-oxide.

Mouse No.	Period of survival (days)	Body weight (g)	Liver weight (g)	Spleen weight (mg)	Lung weight (mg)	No. of nodules in the lung	Ade-noma	Adeno-carcinoma	Metas-tases
1	225, Died					35	+		
2	234, 〃					82	+	+	+*
3	246, 〃					40	+		
4	269, Killed	16	0.8	40		36	+	+	+**
5	285, 〃	20	1.1	230	400	89	+		
6	〃, 〃	20	1.2	80	175	39	+		
7	〃, 〃	20	1.2	84	460	41	+		
8	〃, 〃	27	1.5	90	345	95	+		
9	〃, 〃	26	1.4	115	500	108	+		
10	〃, 〃	24	1.5	120	415	40	+		

* Metastases were found on the diaphragm, along the chest wall, in the lymph nodes in the mediastinal region.

** Metastases were found on the diaphragm and in the lymph nodes in the mediastinal region.

At autopsy, the induced tumors were almost invariably multiple, and both lungs were extensively riddled with these growths. All tumors appeared to grow expansively, but were not encapsulated. Grossly, there was little to differentiate the

adenoma from the adenocarcinoma, as both growths were found intermixed on the surface of the lung. If anything, the adenomas appeared soft, transparent and round nodules. While cancerous growths were often projecting eminently above the plueral surface and with irregular border. And they were opaque, gray in color except when they showed congestion. The most exact criterion for the diagnosis of the cancer was the presence of neoplastic deposits on adjacent tissues. Several deposits of small cancerous masses were found often on the diaphragm, along the chest wall, and in the mediastinal region as the result of adhesion, dissemination or implantation. The lymph nodes lying in the mediastinal region were frequently enlarged with neoplastic growths (Fig. 4).

Microscopically, there was moderate variation in the appearance and in the density of the tumors. The tumor cells were columnar, cuboidal, round and oval. They were arranged in acini, irregular in size and shape, showing papillary formation (Figs. 5~9). These cells occasionally contained droplets of mucus. The nuclei were single, round or oval and varied from lightly to deeply stained. Extensive areas of the lung were replaced by tumors. In a few cases, the advancing border of the infiltrating neoplasm was irregular. Detached tumor deposits occupied spaces of of bronchi or lymphatic canals. Mitotic figures were not numerous. The stroma was usually scanty. Occasionally, tissues were necrotic in the center. Several deposits of tumor in adjacent tissues showed typical patterns of papillary adenocarcinoma rather than those of sarcoma (Figs. 10~12). Then, these points did not follow the description of the metastases of spontaneous carcinoma of the lung in mice described by Wells et al. (17).

The lesion diagnosed as adenoma, occurring in all the mice in the experiment, could readily be mistaken for carcinoma, because of its size and hyperplastic appearance. Althogh the cellular elements of this lesion are hyperchromatic and appear to be adenomatous, they do not exhibit the wide range of variation in their pattern. None of the lesions diagnosed as adenoma yield metastasis outside the lung, despite their large size and number of nodules at autopsy. In the present paper, the classification of adenoma and adenocarcinoma was made under the conservative view point, and the diagnoses are shown in the table.

As is shown in the table, more or less multiple tumor nodules were found in the lung of all mice which survived more than 225 days. And the number of these nodules varied from 35 to 108. Most of them measured from 1 mm to 2.5 mm in diameter while the largest one was 4.5 mm in diameter. In all the cases, findings of the lung were classified adenocarcinoma and adenoma, histologically. It is clearly seen in the table that 2 mice out of 10 were diagnosed as having adenocarcinoma because of the presence of cancerous masses on the diaphragm, or implantations on the chest wall and in the mediastinal region. Organs other than the lung showed no significant

change, and in special, there was no subcutaneous tumor in any of the mice. It is noteworthy that the lung is a sensitive organ in mice when 4-nitroquinoline N-oxide was administered subcutaneously. Spontaneous tumors of the lung are not uncommon in certain strains of mice, and if any, they are usually single. The rarity of spontaneous adenocarcinoma of the lung in animals is certain. Transplantation of a tumor was not tried.

Discussion

What relationship exist between the lesions diagnosed as adenoma and adenocarcinoma in this experiment is open to future investigation. At present, two conceptions are possible on the relationship between them. The one is that, adenoma was first induced by the injections of the carcinogen, and some of them eventually changed into adenocarcinoma in the course of time. The other is that both adenomas and adenocarcinomas were induced simultaneously when the carcinogen was administered, and both would have proliferated into manifestable adenomas and adenocarcinomas independently. Namely, the former view supports that the adenoma is a precancer which may be occasionally progress to adenocarcinoma, and the latter that adenoma and adenocarcinoma have no relation between their origins.

As reported in the previous paper, the full-developed adenoma in mice was definitely induced by a single injection of 4-nitroquinoline N-oxide as early as in three months. However, no carcinoma was found among them when they were sacrificed on the forth month after the beginning of the experiment. Then, it seemed that no carcinoma would have been initiated with a single subcutaneous injection of the carcinogen, or that carcinomas induced by the injection was too early in the stage to be noticed at that time. However, it is not the purpose of the present paper to decide the merits of the two conceptions, for which purpose the data presented are inadequate.

Another difficult point of interpretation in this study is the type of cell giving rise to the tumors. That it is usually derived from the alveoli is certain in the majority of preparations, as many previous investigators observed in the cases of urethane, hydrocarbons and other chemicals. While, the hyperplastic findings frequently found in bronchi or bronchioles of the lung should not escape our notice.

On the other hand, the mechanism of action of 4-nitroquinoline N-oxide on the lung is not clear at present. Two possibilities may be considered; i.e., when the carcinogen was injected in the subcutaneous region, a sufficient amount of the carcinogen gets into the lung directly; or that a metabolite of the carcinogen reaches the lung from the site of injection through the lymphatics or the blood. The first probability is not far-fetched, although much of the carcinogen might react with sulphydryl groups and lose the potencies as has been pointed out by Endo (18) and

Hayashi (19). The possibility of the inhalation of particles can be eliminated in this case as the carcinogen was dispersed in the oil solution. To support the second hypothesis, it is necessary to establish the presence of carcinogenic metabolite of 4-nitroquinoline N-oxide, which is unknown at present.

From the 4-nitroquinoline N-oxide studies performed in this laboratory, lately, it was demonstrated that the incidence of pulmonary tumors in mice is the most remarkable when it was administered subcutaneously than when administered orally, intraperitoneally or externally (20).

Summary

Twenty female mice of dd strain received subcutaneous injections of a total amount of 2.5 mg of 4-nitroquinoline N-oxide in 10 divided dosis. In all 10 mice survived more than 225 days, multiple pulmonary tumors were induced. Most of the tumors were designated as cystadenomas, histologically. In two cases among them, well-established adenocarcinomas with metastases were found. The result indicates that 4-nitroquinoline N-oxide may have distant carcinogenic action from the site of the injection.

Explanation of Plates XXII~XXIV

Fig. 1. Dorsal view of the lung of mouse No. 2, died on the 234 th day. A large nodule of adenocarcinoma is seen.

Fig. 2. Ventral view of the lung of the same mouse. Several nodules of adenocarcinoma are seen. Cancerous deposits (arrow) on the diaphragm are seen at lower part.

Fig. 3. Cancerous deposits (arrow) on the chest wall of the same mouse.

Fig. 4. Dorsal view of the lung of mouse No. 4, which was killed on the 269 th day, showing an enlarged lymph node (arrow). Several nodules are diagnosed as adenocarcinoma histologically.

Fig. 5. An example of the usual type of adenocarcinomas induced. Mouse No. 2.

Figs. 6 and 7. Other types of adenocarcinomas induced. Mouse No. 4.

Figs 8 and 9. Higher magnification of tumors.

Fig. 10. Cancerous deposits on the diaphragm. Mouse No. 2.

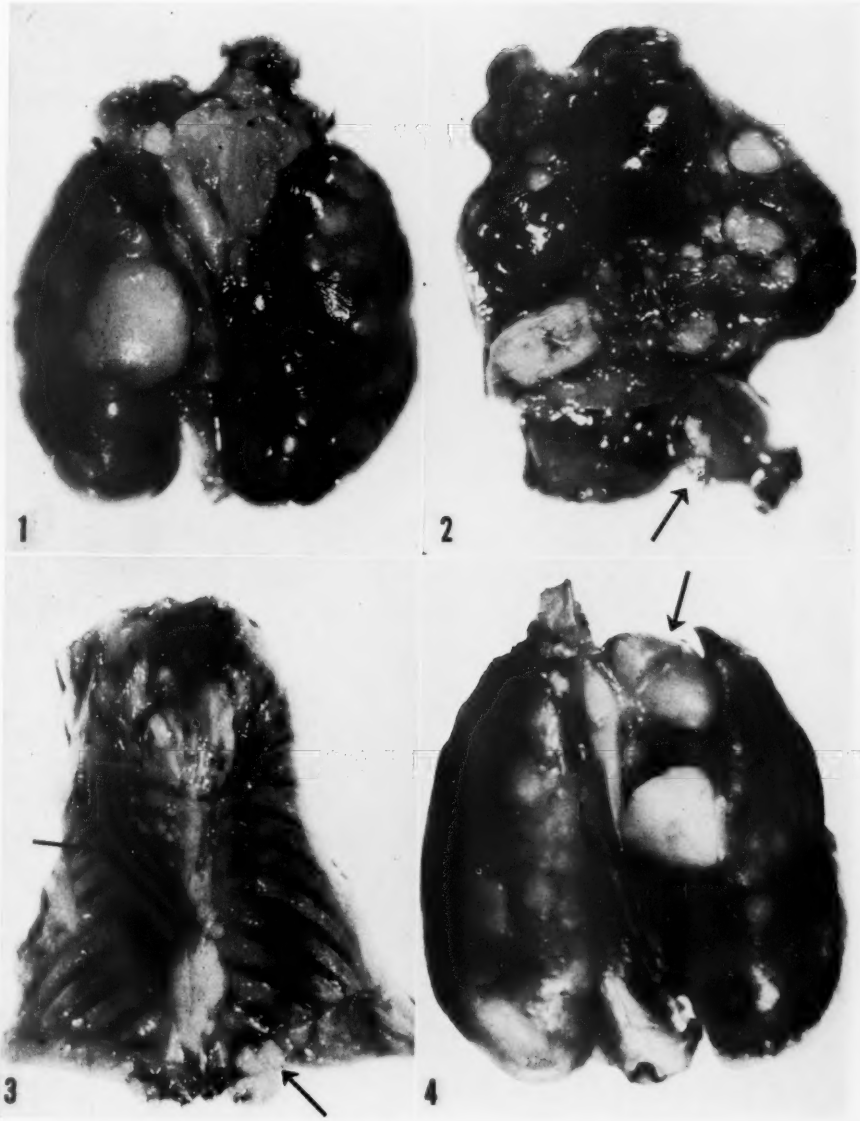
Fig. 11. Cancerous growths into the chest wall. Mouse No. 2.

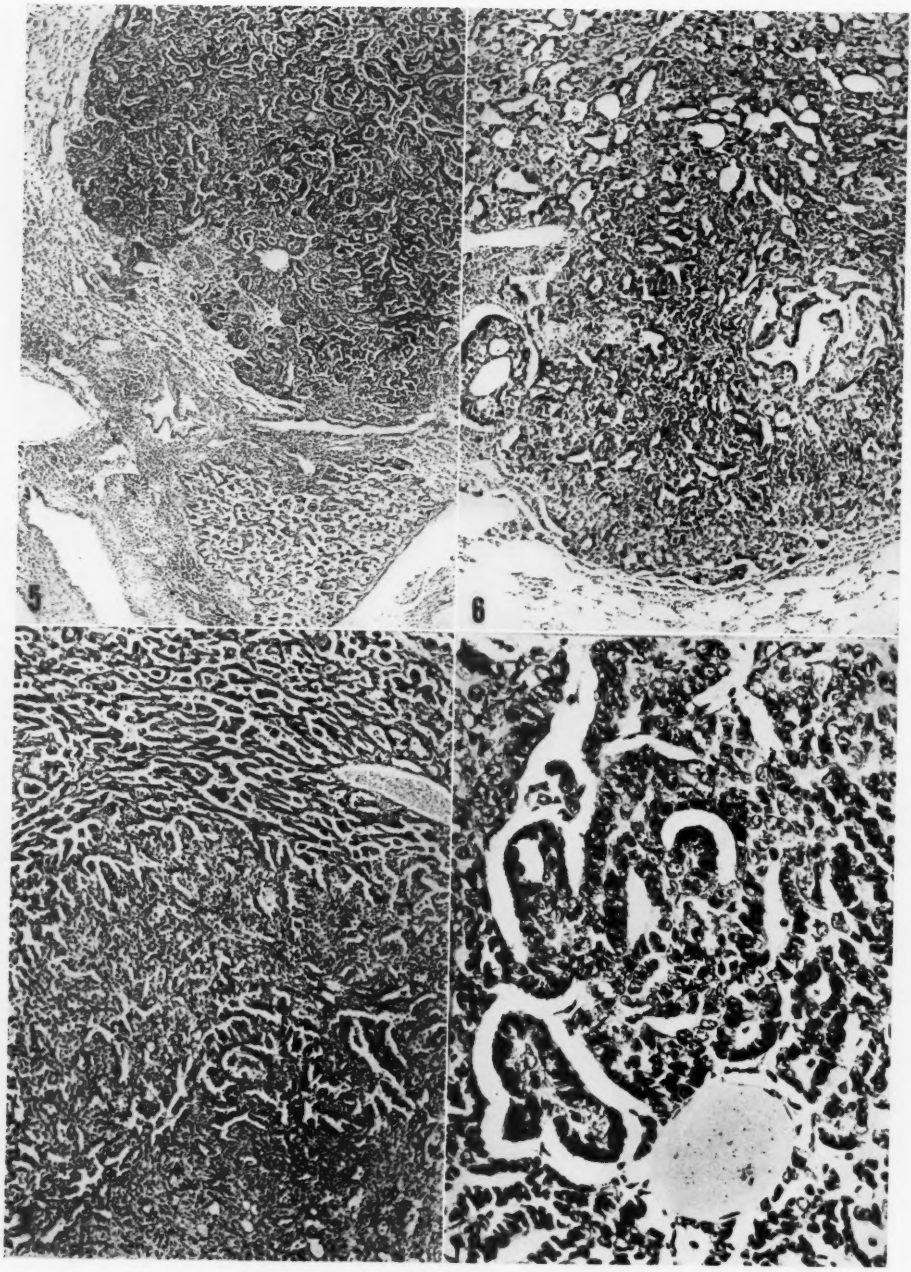
Fig. 12. Implantation of cancer masses in the mediastinal region. Mouse No. 2.

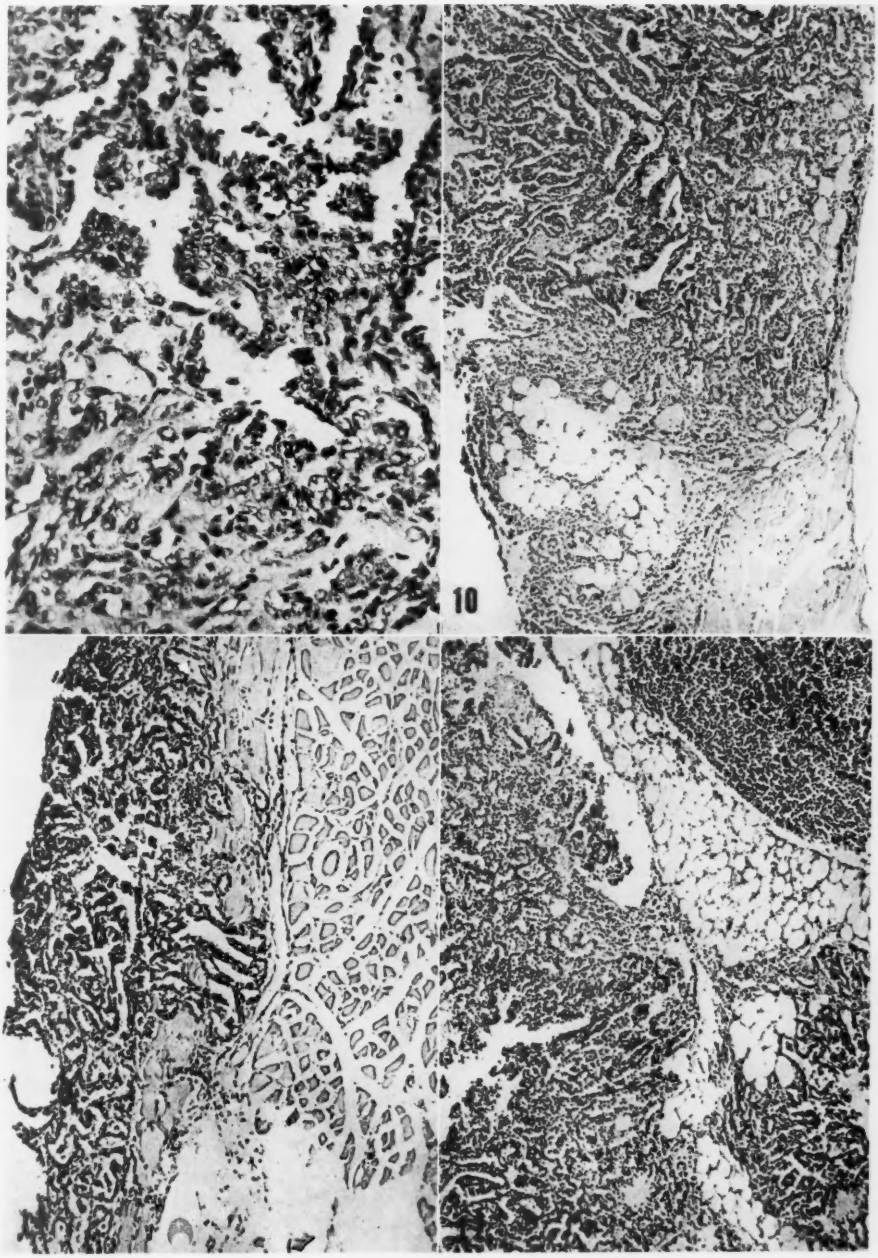
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[GANN, 52, 271~276; December, 1961]

EFFECTS OF FOWL SARCOMA VIRUS ON P^{32} INCORPORATION INTO NUCLEIC ACID IN CHICKEN THYMUS GLAND

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Introduction

Although many reports on the differences between normal and malignant tissues have been presented, information is scanty as to biochemical changes in the course of tumorigenesis, because experimental tumors require long latent periods for tumor induction by the external carcinogens. On the other hand, some virus is known to elicit malignant tumors within a matter of days after injection into susceptible hosts. The virus of chicken sarcoma, Chiba's strain (1), induces tumor in several days when cell free extracts were inoculated into the wing web or thymus gland. Electron microscopic studies demonstrated that the virus content of the tumor originating from the thymus gland was higher than from the other tissue (2).

It was felt that a detailed study of the rate of turnover of P^{32} in the RNA and DNA during the course of tumor induction by this RNA type virus (3) injected into the thymus of fowl would shed some light on the host-virus relation during carcinogenesis. Such *in vitro* studies of P^{32} turnover have been carried out by Hecht and Potter using regenerating liver slices (4). Similar studies have been made by Levy using leucaemia virus (5), and by Cornatzer *et al.* using rabbit papilloma virus (6) during tumorigenesis.

This report presents the effect of sarcoma virus on the uptake of P^{32} into the nucleic acid of chicken thymus gland *in vitro*, day by day, from the 2nd to 9th day after inoculation.

Materials and Methods

Animals: White Leghorn cock of 2 to 3 months old from the same hatchery were used.

Source of tumor: The virus, kindly given to us by Prof. Imai (Department of Pathology, Kyushu University) was serially inoculated into the chick thymus glands.

The tumor 14 days after inoculations was homogenized with equal volume of 0.154 M KCl (pH 7.2) by Waring blender for 4 min. at 0°C. The homogenate was centri-

The following abbrevations are used in this paper: NA, nucleic acid; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; PCA, perchloric pacid.

fuged at 4000 rpm for 5 min.. The resultant supernatant was used as a standard inoculum, which has about 2×10^6 infectivity units per ml.

Virus-Inoculation into thymus glands: Four thymus glands on the left side were exposed by an incision of from 5 cm to 10 cm in length. 0.05 ml. of inoculum was injected into each gland on the left side, and the wound was sutured.

Preparation and incubation of tissue slice: The thymus glands were excised immediately after decapitation of the cock previously inoculated with the virus, and immersed in ice cold saline. The glands from the other side were used for the "virus-free" controls.

The thymus glands, weighing 200–400 mg, were sliced with scissors and pressed on glass plate. The slices were placed into 50 ml. ErlenMeyer flasks, to which had been added 2 ml of Robinson's glycylglycine buffer medium (pH 7.8) containing about 5 μc per ml of inorganic P^{32}. The gas phase was filled with pure O_2. The incubation was carried out at 37°C, and the flasks were shaken about 80 cycles per min. for 1 or 2 hours.

Analytical methods: The incubation was terminated by chilling the slices in ice water, adding 2 ml of ice-cold 10% PCA. Extraction of nucleic acid was performed by the method developed by Shibatani (7) from that described by Key, Simons and Dounce (8). RNA and DNA contents were determined by measuring the absorption at 260 mμ (RNA), and 267 mμ (DNA) by the spectrophotometer (HITACHI) using the coefficients 33.16 for RNA (μg) and 32.94 for DNA (μg) presented by De Deken-Grenson, and De Deken (9). The radio-activities of the RNA and DNA fractions were determined on the metal dish 20 mm in diameter by the Geiger-Müller counter.

Results

Table 1. P^{32} Uptake into the Slice of Normal Fowl Thymus Gland.

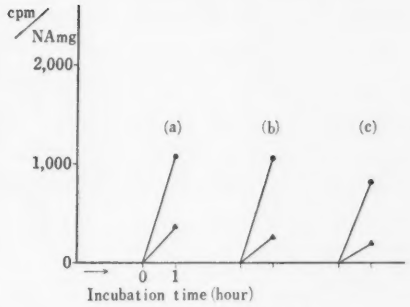
Chicks (White Leghorn)	Age	P^{32} Uptake	
		RNA	DNA
	months	cpm/ug	cmp/ug
1	2-3	11.7	5.32
2	〃	14.2	6.78
2′	〃	15.3	7.00
3	〃	13.1	7.43
3′	〃	12.9	7.34
4	1	11.1	4.31
5	1	17.5	6.42

In 2′ and 3′ experiments, thymus tissues were derived from opposite sides of chiks 1 and 2 respectivaly. Incubation for 2 hours.

P^{32} uptake by normal thymus gland. P^{32} uptake into RNA and DNA of the normal chicken thymus is shown in Table I, in which it can be seen that the P^{32} uptake into RNA and DNA ranged from 11.1 to 17.5 cpm/μg N.A. respectively. Incorporation rate into the thymus of both sides of the same individual chicks showed almost the same values.

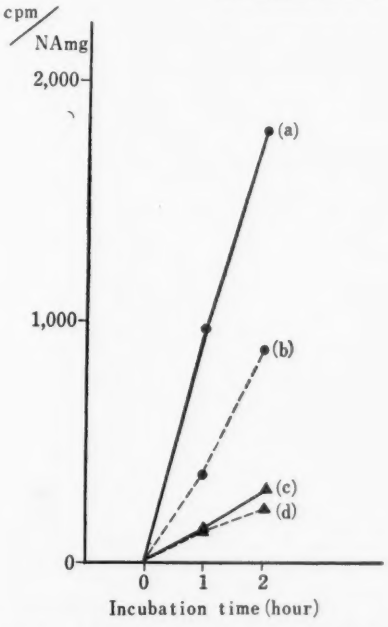
Effect of sampling. The tumor is not homogeneous, but a mixture of solid portion, rudimentary thymus tissues

and jelly-like portion. By the 6 th day following virus infection, the surrounding part of thymus glands was found to be mucous. Accordingly, it is necessary to examine the difference of the incorporation of P^{32} into these portions. Fig. 1 shows that the jelly-like portion has a low specific activity of nu cleic acid compared with the other portions, but that is not so low as expected, for the mucous portion seem to be almost degenerative.



Specific activity of RNA
Specific activity of DNA
(a) Solid portion, (b) Rudimentary thymus gland, (c) Jelly-like portion.

Fig. 1 The Difference of tumor portions on the uptake of P^{32}.



(a) Specific activity of RNA, without Hyaluronidase
(b) Specific activity of RNA, with Hyaluronidase
(c) Specific activity of DNA, without Hyaluronidase
(d) Specific activitv of DNA, with Hyaluronidase

Fig. 2 Effect of Hyaluronidase.

Effects of Hyaluronidase: The jelly-like portion of the tumor can be removed by hyaluronidase. When tumor tissue contained mucous substances, chopped tissue slices tend to aggregate into a mucous mass which is supposed to prevent gas exchange between cell surface and air during the incubation. Such a formation of aggregate can be prevented by removing the jelly-like portion.

Use of hyaluronidase, however, suppresses the incorporation of P^{32} into the nucleic acid of the tumor (Fig. 2). Therefore, this enzyme was not used in the following experiment.

Effects of virus inoculation: Fig. 3 shows the uptake of P^{32} into the normal thymus and the infected thymus. In experiment A, the uptake of P^{32} by the infected thymus glands was compared with the uninfected glands on the opposite side, on the 2nd, 4 th, and 6 th day after inoculation of the virus.

Specific activites of RNA from the infected glands were lower than from the normal glands, except the value on the 2nd day. That of DNA also showed similar tendency as the RNA.

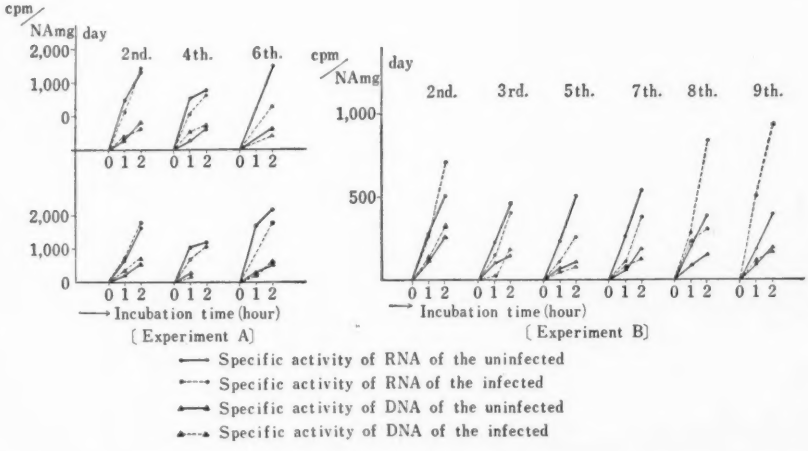


Fig. 3 P^{32} uptake of nucleic acid in the infected and uninfected chicken thymus glands.

In experiment B, P^{32} uptake into the thymus was investigated on the 2nd, 3rd, 5 th, 8 th, and 9 th day after inoculation. From the 3rd to the 5 th day, each infected gland, also, had a low specific activity of RNA and DNA fractions compared with the uninfected. On the 8 th day, however, the specific activity of the infected suddenly increased and became higher than the uninfected.

Changes in the specific activity of nucleic acid fractions from the infected glands are summarized in the Fig. 4. This indicates the incorporation decreases from the 3rd to 7 th day in the infected gland, on the contrary, the infected on the 8 th and

9th day increases to about twice that of the uninfected.

This change in the specific activities of DNA fraction, is also accompanied by that of RNA. There is a parallel between the curve of P^{32} uptake into DNA and RNA.

During the course of investigations, a part of the same glands, used in the incorporation experiment B was taken and histologically examined, which showed that tumor cells first appeared on about the 5th day, and became so numerous that they dominantly occupied the gland on about 7th or 8th day after inoculation.

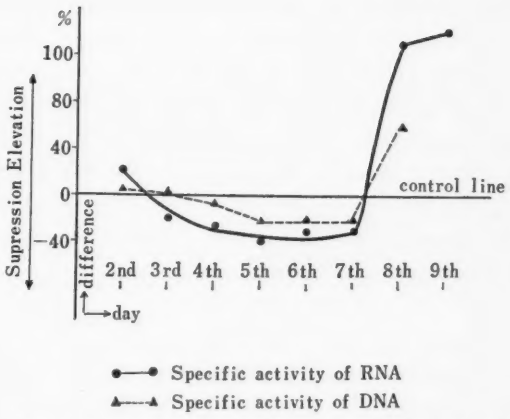


Fig. 4 Difference of P^{32} uptake between the infected and the uninfected glands.

Discussion

During the course of tumorigenesis by the fowl sarcoma virus, an increase in P^{32} uptake into RNA was observed on the 7th day following virus infection (Fig. 4).

Hecht and Potter described that RNA synthesis preceded the beginning of DNA synthesis based on the experiment of incorporation of C^{14}-orotic acid into RNA and DNA of regenerating rat liver slice (4). In our experiments the preceding RNA synthesis was not observed. This discrepancy is not clearly explained at the present time. This is probably due to the difference between normally controled tissues such as liver and unlimitedly growing malignant tumors, or to our assay system which could not catch this peculiar RNA synthesis.

The parallelism between RNA synthesis and DNA synthesis provided evidence to support possibility that there is no expressin of RNA synthesis related to the virus multiplication, except the expression of tumor cell reproduction. Rafelson *et al.* (12) described the effects of Theiler GD virus infection on P^{32} uptake by mouse brain. They concluded that there was the increase of the specific activity of RNA due to multiplication of virus, because of no increase of the specific activity of DNA.

Electron microscopic studies on the fowl sarcoma induced by Chiba's strain (2) revealed that in chick thymus gland the virus was not demonstrated until the 3rd day following infection, then the virus begin to appear about 4th day, and virus content reached the maximum on the 6th or 7th day. The time of this maximal virus content is earlier than that of rise of RNA synthesis in our experiments, as

shown in Fig. 4, showing a decrease in the uptake of P^{32} into DNA and RNA from the 3rd to 7 th day following virus infection. The decrease of nucleic acid synthesis is probably related to the degenerative change preceding tumor formation.

Lo *et al.* (10) reported that the tissue culture of normal chicken fibroblasts infected with Rous sarcoma virus became degenerative within 8 to 14 days, and after serial subcultures from these degenerating cells a group of hypertraphied and vigorously growing cells appeared, which then dominantly occupied the culture. Morgan (11), also described the destructive change of chorioallantoic membrane before cellular hyperplasia, using herpes simplex virus.

Summary

P^{32} uptake into DNA and RNA of chicken thymus gland *in vitro* was investigated at different stages of tumorigenesis caused by chicken sarcoma virus (Chiba's strain).

(1) The specific activities for the different portions of tumor were compared. The solid portion and the rudimentary thymus gland were on a similar level and the jelly-like portion was lower.

(2) From the 3rd to 7 th day after virus inoculation, the specific activity of RNA was depressed, and from the 8 th day this value increased compared with that of control. Specific activity of DNA changed also accompanied that of RNA.

From the above findings it was concluded that specific activities of RNA and DNA express cell reproduction, and there was no expression of the activity of RNA and DNA fractions ocurred from the 3rd to 7 th day after virus infection, but the reason of this decrease is at present not clarified.

The authors are grateful to Prof. H. Endo and Associate Prof. T. Takahashi for their helpful suggestions and encouragements. This investigation was aided in part by a grant from the Ministry of Education.

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[GANN, 52, 277~292; December, 1961]

OBSERVATIONS ON THE CRABTREE EFFECT IN EHRLICH ASCITES TUMOR CELLS, SIGNIFICANCE OF BICARBONATE

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It is well known that the addition of glucose causes a fall of respiration in tumor cells and this effect of glucose, known as the Crabtree effect, is peculair to tumor cells, though postnatal retina has been reported as an exception.[1] Apart from the original investigation of Crabtree,[2] however, nearly all of the recent investigations on this problem have been carried out using the reaction media which were buffered with phosphates in the absence of CO_2 in the gas phase. There seems to have been no attempt to explore the possibility that the presence of CO_2 or $NaHCO_3$ in the reaction system may alter the rate of respiration and even the course of the intracellular reactions.

On the other hand, the respiratory mechanism or regulation in tumor cells in presence and absence of glucose has often been scrutinized but within a very early phase, say, several minutes, of incubation period.[3] However, it may not be entirely safe to extrapolate directly the results obtained from short-term experiments to long-term ones.

It has been reported by Warburg et al.[4] that when ascies tumor cells are submitted to incubation suspended in the ascitic fluid which has been fortified with $NaHCO_3$ and glucose and in the presence of CO_2 in the gas phase, a linear rise in positive pressure follows in contrast to negative pressures observed in all of normal tissues examined, and thus normal and tumor cells are simply distinguishable by their manometric behavior. Since it was found in the present investigation that much the same was true when Krebs-Ringer bicarbonate solution was employed in place of the ascitic fluid, it was attempted to determine the metabolic rates also by the usual 'indirect method' of Warburg.

A particular concern in the present investigation is to examine whether the presence of $NaHCO_3$ (or CO_2) is of significance for the metabolism of tumor cells, observation being made over a range of 120 minutes. As shown by the results given below, it was found that the metabolic pattern in tumor cells was different depending upon the presence and absence of $NaHCO_3$. Thus, the presence of $NaHCO_3$ seems to be of special significance for living tumor cells.

Materials and Methods

Preparation of tumor cell suspensions: Ehrlich ascites carcinoma cells were grown in adult, well nourished mice from our stock colony dd D strain; the animals were fed ad libitum on dry chew (the Oriental Yeast Co., Tokyo) and Water. At about 7–10 days after implantation, the ascitic fluid was withdrawn with a pipette (Komagome) after opening the peritoneal cavity. The ascitic fluid was centrifuged at 2000 r.p.m. for 20 minutes at room temperature. The packed cells obtained were suspended in about ten times their volume of plain Krebs-Ringer solution and centrifuged at 800 r.p.m. for 5 minutes to remove blood elements. This washing procedure was repeated three times, followed by recentrifugation at 2000 r.p.m. for 20 minutes. The washed cell pellet freed of erythrocytes was suspended homogenously with aid of a pipette (Komagome) in 9 times its volume of Krebs-Ringer phosphate or Krebs-Ringer bicarbonate solution saturated with oxygen containing 5% CO_2. Cell counts were taken with this cell suspension by use of a haemocytometer. In most cases, the cell concentration was found to fall within a range of $2–6\times10^7$ cells per ml. In manometric experiments, each 1 ml. of the cell suspension was added to 2 ml. of Krebs-Ringer phosphate solution or to 6 ml. of Krebs-Ringer bicarbonate solution, respectively.

Manometric experiments: The incubation media used were Krebs-Ringer phosphate and bicarbonate prepared according to Umbreit et al.[5] Besides the Ringer media, ascitic fluid itself was also employed, which was prepared by the method of Warburg et al.[4], as follows.

Ascitic fluids were removed from five or six tumour-bearing mice about 10 days after implantation. A total of 35 ml. or more of combined fluids was centrifuged at 2,000 r.p.m. for 20 minutes. The clear supernatant was transferred into a stoppered flask and heated at 57° in a small thermostat for 10 minutes, giving rarely a slight amount of protein coagula. After cooling to room temperature, the fluid was recentrifuged at 4,000 r.p.m. for 15 minutes and a colourless or slightly yellowish, clear solution was obtained. Then, the content of $NaHCO_3$ was determined manometrically with 1 ml. of the treated ascitic fluid and those of lactic acid and glucose by the chemical methods with another 1 ml. It was found that the $NaHCO_3$ content was about half that of Krebs-Ringer bicarbonate and the lactate content was of the order of 1000 μg per ml. on an average, while glucose was nearly absent as described by Warburg et al.[4][6] or, if any, of the order of below 50 μg per ml. Thus, the main body of ascitic fluid was fortified by adding measured amount of $NaHCO_3$ in the solid state and gassed with a mixture of 95% O_2–5% CO_2 (v/v), glucose being dissolved in the fortified ascites and added from the side arm during incubation. A washed tumour cell pellet was suspended in nine time its volume of fortified

ascitic fluid and cell counts were taken in the same way as described above.

When oxygen uptake alone was followed, Krebs-Ringer phosphate was employed as the suspension medium. One ml. of the cell suspension was added to 1.5 ml. of Krebs-Ringer phosphate in the main compartment of ordinary Warburg vessel with alkali in the center well and air in the gas phase. Glucose, disolved in 0.5 ml of Krebs-Kinger phosphate, was added from the side arm after the 10-minute period for thermal equilibrium, addition of plain Ringer medium being made in the control vessel. The initial concentration of glucose added was 10^{-2} M in most cases.

When O_2 uptake and CO_2 release were followed simultaneouly, the "indirect" method of Warburg was employed, using, according to Warburg et al.[4)7)], cubiform vessels of different volume containing the same volume of Krebs-Ringer bicarbonate or fortified ascitic fluid. The volumes of paired vessels were about 28 ml. and 19 ml., respectively. To 5.5 ml. of suspension medium placed in each of the vessels was added 1 ml. of the cell suspension, gassed with a mixture of O_2-CO_2 (95 : 5, v/v) and incubated at 38°. After an equiliberation period of 10 minutes, readings were taken carefully by corporation of two people at 5 minute intervals for 30, 60, or 120 minutes. Glucose, dissolved in 0.5 ml. of Krebs-Ringer bicarbonate or ascitic fluid, was added from the side arm 10 minutes after the first reading. Unless otherwise stated, the amount of glucose added was arranged so as to give an initial concentration of $5 \cdot 10^{-3}$M. Thus, the glucose concentration in this case is half that in Krebs-Kinger phosphate but its net amount per cell is roughly equal in both cases.

Chemical analysis: At the end of incubation period, the vessels were detached immediately and appropriate amounts of cell suspension were deproteinized after the method of somogyi.[8)] According to the amounts of glucose and lactate to be expected, the amounts of cell suspensions employed were varied: 1 or 2 ml. in the case of Krebs-Ringer phosphate and 1 to 4 ml. in the case of Krebs-Ringer bicarbonate. With aliquots of the Zn-Ba filtrate obtained from a total of 10 ml. of deproteinizing mixture the determinations of glucose and lactate were run. Lactate was determined by the method of Barker and Summerson.[9)] Glucose was determined by the method of Nelson[10)].

pH: This was determined potentiometrically.

Respiratory quotient: The O_2 uptake and the CO_2 output were determined by the indirect method as mentioned above. At the same time, lactic acid production was determined chemically with one of the two manometric vessels at the end of incubation period and the respiratory CO_2 was computed by subtracting the calculated amount of CO_2 corresponding to lactic acid produced from the total CO_2 output. When changes with time of respiratory quotient were determined, a series of two or three parallel manometric vessels containing the identical amounts of cell suspeinsion and glucose was incubated and at two or three different times during the

experiment vessels were removed from the bath for determination of lactic acid. It has been found that in Krebs-Ringer bicarbonate medium containing $5 \cdot 10^{-3}$M glucose the glycolytic rate of tumor cells was so linearly sustained over a period of two hours that it seemed possible to get intermediate levels of lactic acid on a basis of its single determination at the end of incubation period. In the absence of glucose added (endogenous metabolism) where next to no glycolysis occurs, respiratory quotient was given directly as a ratio of total CO_2 output to O_2 uptake.

Expression of experimental data: Unless otherwise stated, these were expressed as μg or μl per 10^6 cells throughout.

Results

Crabtree effect in Krebs-Ringer phosphate and its dependence on glucose concentration. It has been observed by a host of workers[11)12)13)14)] that the Crabtree effect appears clearly when glucose is added at high concentrations, but indefinitely or even inversely at lower concentrations such as 10^{-3}M or less. This is true when long-term experiments are carried out using the suspension medium to which glucose was initially added. At lower concentrations of glucose, it is to be considered that the Crabtree effect had been nearly terminated at the time of taking the reading, because, as seen below, for $2 \cdot 10^7$ tumor cells per ml. 20 minutes incubation is sufficient for glycolising completely 10^{-3} μM of glucose. In such cases, therefore, the respiration which is observed is not based on glucose as substrate but on lactate formed by glycolysis, the presence of which rather enhances the endogenous respiration.

Accordingly, in order to know whether the Crabtree effect is dependent on the concentration of glucose added or not, varying amounts of glucose were added from the side arm after the thermal equilibration had been reached and the whole time course of the Crabtree effect was followed. At the same time, the glucose utilization and lactate formation were determined at graded intervals with another series of reaction vessels. The results are presented in Fig 1. In Fig. 1, B, values in the ordinate show the oxygen uptakes at intervals of five minutes, the solid and clear bars representing the values in presence and absence of glucose, respectively. This shows the time course of the Crabtree effect more clearly than the accepted representation of plotting total respiration and time.

The results in Fig. 1, A and B, show that the Crabtree effect occurs irrespective of the concentration of glucose added but its durability is dependent on the glucose concentration. When glucose was added at a high concentration of 10^{-2}M the respiration falls promptly and the depressed rate of respiration is sustained over an incubation period of 50 minutes (Fig. 1, B). Under the same conditions, lactate formation and glucose utilization proceed rapidly but glucose still remains at a level of

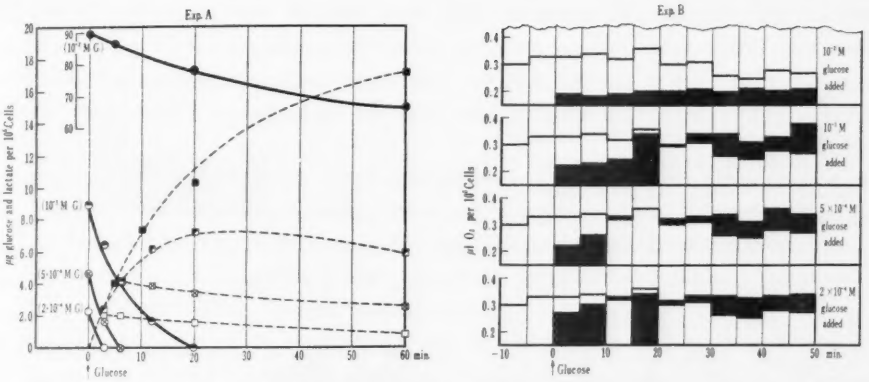


Fig. 1. Time courses of glucose utilization, lactate formation and oxygen consumption in Ehrlich ascites tumor cells.

Washed cell suspensions were prepared in the way as described in the text. Experiments A and B were carried out with separate cell suspensions, the cell concentrations of which were 6.0×10^7 cells per ml. and 5.7×10^7 cells per ml., respectively. In Exp. A, 6 ml. portions of the washed cell suspension were added to 50 ml. Erlenmyer flasks containing 9 ml. of Krebs-Ringer phosphate solution (pH 7.4), stoppered and incubated at 38° with shaking. In Exp. B, 1 ml. portions of another cell suspension were put in Warburg vessels containing 1.5 ml. of Krebs Ringer phosphate solution and incubated with air in the gas phase. After the 10 min. period of temperature equilibration, calculated amounts of glucose dissolved in 3 ml. of Krebs-Ringer phosphate solution were added with pipettes in Exp. A, but in Exp. B, preliminary manometric readings were made for 10 minutes and then 0.5 ml. of the glucose solutions or the plain saline (control) were added from the sidearms (zero time). The final volumes of cell suspension were thus 18 ml. in Exp. A and 3 ml. in Exp. B, but the cell concentration was approximately the same in both cases, i.e., 2.0×10^7 cells per ml. in Exp. A and 1.9×10^7 cells per ml. in Exp. B. In Exp. A, 3 ml. portions of the reaction mixture were pipetted out at intervals of 3, 6, 12, 20 and 60 minutes, added to 0.5 ml. of 3 N H_2SO_4 and submitted to the determination of glucose and lactate. In Exp. B, oxygen uptake was measured carefully at 5-min. intervals.

In Exp. A, solid lines represent the glucose utilization and dotted lines the lactate formation, a separate scale being given for the glucose utilization at a high glucose concentration of 10^{-2}M.

● :- glucose and ■ :- lactace levels at the addion of 10^{-2}M glucos ;
◓ :- 〃 ◪ :- 〃 10^{-3}M 〃 ;
⊗ :- 〃 ⊠ :- 〃 5×10^{-4}M 〃 ;
○ :- 〃 □ :- 〃 2×10^{-4}M 〃 ;

In Exp. B, values in the ordinate show the oxygen uptakes at intervals of five minutes, the solid and clear bars representing the values in presence (Crabtree effect) and absence of glucose, respectively. For clarity, time courses of respiration at different glucose concentrations are given separately in comparison with that of one and the same endogenous respiration.

4000 μg per vessel or above at the end of incubation (Fig. 1, A). When 10^{-3}M glucose was added, it is exhausted after about 20 minutes of incubation; in agreement with this, the respiration which has been suppressed clearly for initial 15 minutes is restored to the endogenous level at the 4 th five-minute interval. When the initial concentrations of glucose were 5.10^{-4}M, and 2.10^{-4}M, they were consumed completely within about 6 and 3 minutes, respectively. Even in such cases, the inhibition of respiration occurred distinctly at early periods of incubation. The suppressed respirations, however, is restored sooner to the endogenous level, namely, after about 10 and 5 minutes respectively, corresponding to the time of exhaution of glucose added. The respiration which has been released from the Crabtree effect

shows a rather higher and sustained rate than that of endogenous respiration. Presumably, this increase of respiration may be due to the oxidation of lactate formed by glycolysis, in view of the fact that at lower concentrations of glucose glycolysis reaches steeply a maximum synchronized with the exhaution of glucose and falls slowly thereafter.

It must be noted that even when a sufficient amount of glucose (10^{-2}M) is present, glycolytic rate which was very rapid at the early phases passes soon into slow with time. This rapid decline of glycolytic rate, though partly due to the fall of pH, seems to be a metabolic character of tumor cells conditioned by Krebs-Ringer phosphate. As will be shown also in the succeeding report, this is contrasted with that which is displayed when Krebs-Ringer bicarbonate is used instead, where glycolysis proceeds linealy throughout the incubation period.

Lactate and pyruvate can stabilize or rather enhance the respiration of tumor cells. But the Crabtree effect occurs clearly indifferent to the presence of these metabolites. For instance, the respiration stabilized by 10^{-2}M lactate is simply depressed by adding 10^{-2}M or 10^{-3}M glucose as well (Fig. 2 and Fig. 3). There seems to be no competition between two kinds of substrate, glucose being always dominant over lactate and pyruvate in tnis respect (Fig. 4).

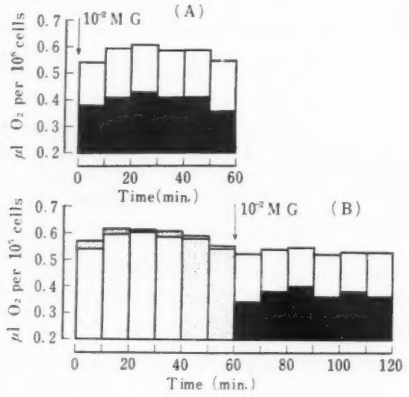


Fig. 2. Inhibitory effect of glucose (10^{-2} M) on lactate respiration of ascites tumor cells in Krebs-Ringer phosphate solution.

Experimental procedures are the same as those in Fig. 1, provided that the suspension medium contained initially 10^{-2}M lactate. The addition of glucose (arrows) was made at the zero time (A) and 60 minutes (B) of incubation period. Clear and dotted bars indicate the oxygen uptakes as control at intervals of ten minutes and solid bars those after glucose addition. In (B), glucose was added to the series of dotted bars.

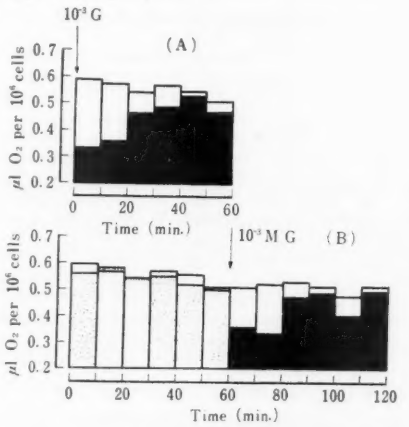


Fig. 3. Inhibitory effect of glucose (10^{-3} M) on lactate respiration of ascites tumor cells in Krebs-Ringer phosphate solution.

Experimental procedures are the same as those in Fig. 2. Lactate concentration, 10^{-2}M. The addition of glucose was made at the time shown by arrows. Clear and dotted bars indicate the oxygen uptakes as control at intervals of 10 minutes and solid bars those after glucose addition. In (B), glucose was added to the series of dotted bars.

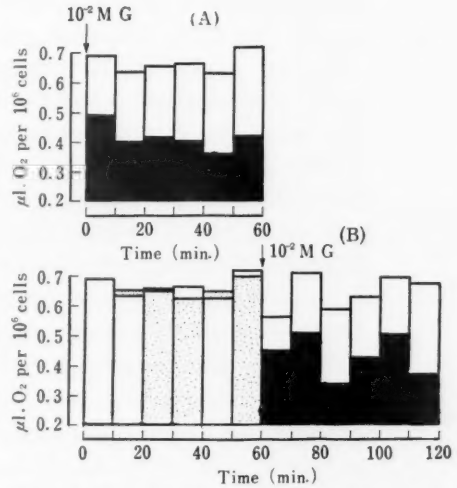


Fig. 4. Inhibitory effect of glucose (10^{-2}M) on pyruvate respiration of ascites tumor cells in Krebs-Ringer phosphate solution.

Experimental procedures are the same as those in Fig. 1, providing that the suspension medium contained initially 10^{-2}M pyruvate. The addition of glucose was made at the time shown by arrows. Clear and dotted bars indicate the oxygen uptakes as control at intervals of 10 minutes and solid bars those after glucose addition. In (B), glucose was added to the series of dotted bars.

On the face of these facts, it seems reasonable to say that when Krebs-Ringer phosphate is the suspension medium, the respiration of tumor cells is promptly lowered by the addition of glucose irrespective of its concentration (Crabtree effect) and the lowered repsiration is sustained as long as glucose remains but its original endogenous level is restored with the exhaustion of glucose added.

Manometric studies of carbohydrate metabolism in ascitic fluid and in Krebs-Ringer bicarbonate. It is beyond doubt that Krebs-Ringer phosphate, though accepted as usual medium, is far from physiological for living tumor cells; Krebs-Ringer bicarbonate is better and homologous ascitic fluid itself is best in this sense.

Warburg et al.[4] have employed ascitic fluid and serum, which had been adequately treated and fortified, as suspension media for ascitic tumor cells and normal tissues, respectively, with a mixture of O_2–CO_2 (95 : 5, v/v) in the gas phase and followed the overall gas exchanges. They found that in the presence of glucose and if manometric vessels of adequate and nearly uniform volume were employed, a linear rise of positive pressure occurred in tumor cells, while that of negative pressure in all of normal tissues examined. From this, they have concluded that tumor cells can be distinguished from normal tissues by the metabolic pattern of carbohydrate which reflects manometrically the overall gas exchange.

This metabolic phenomenon peculair to tumor cells has been re-examined according essentially to the method of Warburg et al.[4], except that different amounts of glucose were added from the side arm at the zero time of incubation (10 minutes after the first reading) and the total gas exchange was followed for 120 minutes. The results are presented in Fig. 5.

It was found that as reported by Warburg et al.[4], a linear rate of increase in

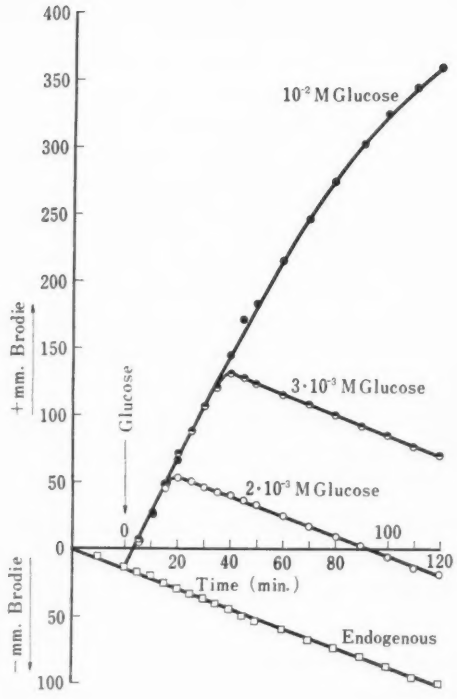


Fig. 5. Overall gas exchange of ascites tumor cells in homologous ascitic fluid.

Ascitic fluid was treated appropriately and fortified with $NaHCO_3$ after Warburg and gassed with a mixture of 95% O_2-5% CO_2 (v/v). 1.5 ml. of this ascitic fluid were placed in the manometric vessel to which was added 1 ml. of cell suspension in the same ascitic fluid, gassed and incubated at 38°. After temperature equilibration, measured amounts of glucose dissolved in 0.5 ml. of ascitic fluid were added from the side-arm at the zero time of experiment (arrow). The number of cells was 37.6×10^6 per vessel and the volume of vessels was of the order of 20 ml.

positive pressure occurred when glucose was added at a high concentration of 10^{-2} M, but under endogenous conditions where there was no addition of glucose negative pressure appeared from start and followed a linear course in the same way as in normal tissues. It is interesting to note that when smaller amounts of glucose ($3 \cdot 10^{-3}$M and $2 \cdot 10^{-3}$M) were added, it happened in a short time that ascending positive pressure turned abruptly to negative pressure and ran its linear course parallel with that of the endogenous metabolism. Attaining the turning point from positive to negative pressure comes the sooner, the less the amount of glucose added, indicating that the presence of glucose is a limiting factor in this phenomenon. This may also be correlated with the observational facts of the Crabtree effect shown in Fig. 1. It must further be noted that when a sufficient amount of glucose (10^{-2}M) is added, a rise of Brodie maintains a linear rate for initial 60 minutes or so, just as described by Warburg et al. but passes to slower rates with time during the following 60 minutes, suggesting that the overall gas exchange disturbs its balance by the predominance of negative pressure which might be due to an increase in oxygen uptake.

A quite similar pattern of overall gas exchange was obtained when Krebs-Ringer bicarbonate was employed in place of the fortified ascitic fluid, as seen from Fig. 6. From this, it follows that so long as the overall gas exchange is concerned, Krebs-Ringer bicarbonate can replace the ascitic fluid and thus may be more physiological for tumor cells than Krebs-Ringer phosphate.

Oxygen uptake and total CO_2 output determined by the 'indirect method' of Warburg.

Since the fortified ascitic fluid and Krebs-Ringer bicarbonate are equivalent in determining the overall gas exchange of tumor cells, it is also possible to determine with ease the respiration by the 'indirect method' of Warburg and to follow the time course or pattern of the Crabtree effect in the presence of $NaHCO_3$.

Two results of typical experiments are given in Fig. 7 and Fig. 8. Tumor cells used were 9 and 14 days old after implantation, respectively.

It is distinctly worthy of notice that there is a marked difference between the patterns of the Crabtree effect in the presence and absence of bicarbonate. As seen above (Fig. 1), in the absence of bicarbonate the lowered rate by glucose of respiration is sustained linearly until glucose added is exhausted. In the presence of bicarbonate, however, the Crabtree effect which appears distinctly at the early phase of incubation disappears and goes into reverse with time, even if a sufficient amount of glucose still remains in the reaction system. That is, when Krebs-Ringer bicarbonate containing $5 \cdot 10^{-3}$M glucose is the suspension medium, the depressed rate of respiration is not only released from the inhibitory action of glucose within 60 minutes or less but it continues to increase with time and even goes beyond that of endogenous respiration, resulting thus in a reversal of the Crabtree effect. The time of release from glucose inhibition appears to depend on the age of tumor cells.

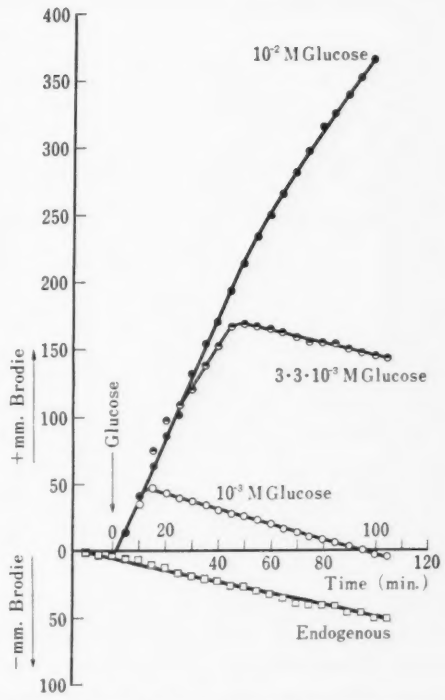


Fig. 6. Overall gas exchange of ascites tumor cells in Krebs-Ringer bicarbonate solution.

Experimental procedures are quite the same as in Fig. 5, provided that Krebs-Ringer bicarbonate solution was employed in place of ascitic fluid. The number of cells was 35.0×10^6 per vessel.

Since nearly no formation of lactic acid occurs in endogenous metabolism, if it is assumed that the total CO_2 output is derived from the respiratory CO_2, then the respiratory quotients obtained were of the order of 0.7 on an average (Table 1), showing that in the endogenous respiration fats are the principal substrate in agreement with the findings of Medes and Weinhouse[15]. As contrasted with this, it must

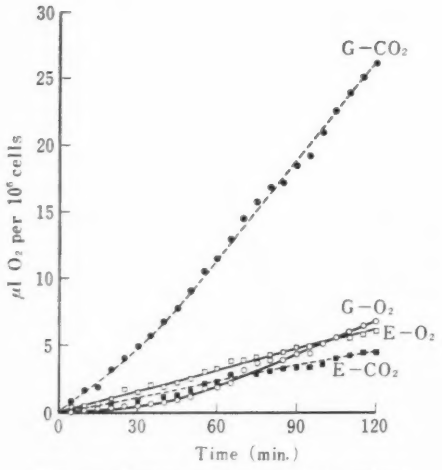


Fig. 7. Oxygen uptake and CO_2 output of ascites tumor cells in the presence of bicarbonate-(9-day-old cells).

According to Warburg,[2)7)] the same volume of incubation fluid of 7 ml. was used, employing a pair of cubifrom vessels of different volume. The initial concentration of glucose added was $5 \cdot 10^{-3}$M. The number of cells was 33.4×10^6 per vessel. Experimental details are given in the section of Materials and Methods.

G-O_2, O_2 uptake and G-CO_2, CO_2 output in the presence of glucose.

E-O_2, O_2 uptake and E-CO_2, CO_2 output in the absence of glucose (endogenous).

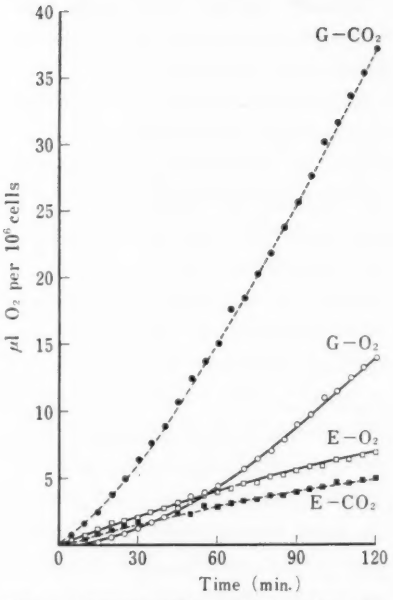


Fig. 8. Oxygen uptake and CO_2 output of ascites tumor cells in the presence of bicarbonate-(14-day-old cells).

Experimental procedures are the same as those in Fig. 7, provided that the number of cells was 34.2×10^6 per vessel.

G-O_2, O_2 uptake and G-CO_2, CO_2 output in the presence of glucose.

E-O_2, O_2 uptake and E-CO_2, CO_2 output in the absence of glucose (endogenous).

be noted that when glucose is present the respiratory quotients are always more than unity (Table 1). Much the same has been reported by Kun et al.[16)] and Bloch-Frankenthal and Weinhouse[17)29)] even when Krebs-Ringer phosphate was the suspension medium. It must further be added that in Krebs-Ringer bicarbonate the pH value is kept fairly constant, namely, at the end of two hours' incubation it is 7.2 or 7.3 in the presence and 7.5 or 7.6 in the absence of glucose, in contrast to Krebs-Ringer phosphate where it falls usually below 7 at the end of experiment.

Respiration determined by the 'direct method' of Warburg in the presence of bicarbonate.

As shown above, when long-term observation was made, the pattern of the Crabtree effect is different depending upon the presence and absence of $NaHCO_3$ in the sus-

Table 1. Metabolic rates and respiratory quotients of ascites tumor cells in the presence and absence of glucose.

The results in Fig. 7 and Fig. 8 are summed up from another aspect. Lactic acid production was determined chemically at 60 and 120 minutes of incubation period and is expressed in terms of CO_2 evolved (glycolytic CO_2 is given as the difference between total and glycolytic CO_2 s. In endogenous metabolism, glycolytic CO_2 was assumed to be zero.

Growth period of Cells (day)	Time interval (min)	No glucose (endogenous)					With glucose (5×10^{-3}M)					Remarks
		O_2	Total CO_2	Glycolytic CO_2	Respiratory CO_2	R.Q.	O_2	Total CO_2	Glycolytic CO_2	Respiratory CO_2	R.R.	
		(μl. per 10^6 Cells)					(μl. per 10^6 Cells)					
9	0~30	−1.62	1.01	—	1.01	0.62	−0.54	4.99	—	—	—	Fig. 7
	0~60	−3.34	2.29	—	2.29	0.69	−2.08	11.38	9.08	2.30	1.16	
	60~120	−2.86	2.34	—	2.34	0.82	−4.69	14.73	9.21	5.52	1.18	
14	0~30	−2.05	1.55	—	1.55	0.76	−1.34	6.39	—	—	—	Fig. 8
	0~60	−3.88	2.82	—	2.82	0.73	−4.36	15.00	10.40	4.60	1.06	
	60~120	−3.04	2.23	—	2.23	0.73	−9.62	22.15	10.56	11.59	1.21	

pension medium. The importance of $NaHCO_3$ can also be realized, though roughly, by the usual 'direct method' of Warburg, using the ascitic fluid fortified with bicarbonate or Krebs-Ringer bicarbonate as suspension media. Namely, 2.5 ml portions of tumor cell suspension were placed as usual in conical manometric vessels with KOH in the center well and air in the gas phase, and incubated. After 20 minutes of endogenous respiration, definite amounts of glucose dissolved in 0.5 ml. of ascitic fluid were added from the side-arm and the respiratory rates were followed further over a range of 120 minutes. The results are given in Fig. 9 and Fig. 10, where ascitic fluid and Krebs-Ringer bicarbonate are the suspension media, respectively.

In Fig. 9, it is shown that at low concentrations of glucose added such as 10^{-3}M and 4×10^{-3}M the initial fall of respiration is restored to the endogenous level at the time of exhaution of glucose, showing thus a similar pattern of the Crabtree effect to that in Krebs-Ringer phosphate; whereas a high concentration of glucose (10^{-2}M), even if it also causes a distinct fall of respiration, cannot sustain its inhibitory action throughout in spite there of remaining still a sufficient amount of glucose, namely, after about 60 minutes of incubation the respiration is released from inhibition and follows steeply an ascending curve. The same phenomenon was observed when Krebs-Ringer bicarbonate was used in place of ascitic fluid (Fig. 10).

As a matter of course, in these experiments where there is no CO_2 in the gas phase the pH values of reaction media are to be changeable as incubation proceeds. Ascitic

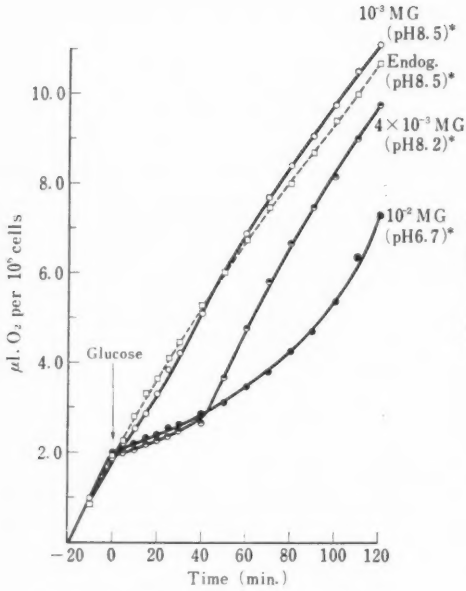


Fig. 9. Pattern of the Crabtree effect in ascitic fluid obtained by use of usual 'direct method'.

Ascites tumor cells were suspended in 2.5 ml. of ascitic fluid fortified with $NaHCO_3$, the cell concentration being 37.0×10^6 cells per vessel, and incubated with KOH in the center well and O_2 in the gas phase. Varying amounts of glucose dissolved in 0.5 ml. of the ascitic fluid were added from the side-arm at the time shown by an arow. Experimental details are in the text.

* pH of the suspension media at the end of experiment.

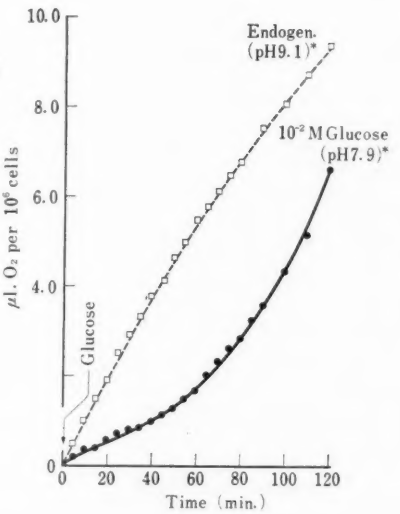


Fig. 10. Pattern of the Crabtree effect in Krebs-Ringer bicarbonate obtained by use of usual 'direct method'.

Experimental procedures are quite the same as those in Fig. 9, provided that the cell concentration was 32.0×10^6 cells per vessel and the addition of glucose was made at the zero time of experiment (arrow).

* pH of the suspension media at the end of experiment.

fluid which presents a well-buffered medium in itself shows a relatively small change of pH. However, in the case of Krebs-Ringer bicarbonate, especially, in endogenous metabolism where no lactic acid is formed, pH shifts to as high a value as 9 at the end of incubation owing to the decomposition of bicarbonate; neverthless, the characteristic pattern of the Crabtree effect seems to be reproducible as long as bicarbonate is present

Discussion

The pattern of the Crabtree effect. It is worth while indeed to note that the time course of the Crabtree effect or its pattern is modified by the presence of bicarbonate, or to say in general, the carbohydrate metabolism of ascites tumor cells is greatly affected by the presence of bicarbonate.

Apart from the original investigation of Crabtree,[2] nearly all of recent investigations on the Crabtree effect have been carried out in the media containig no bicarbonate and thus no CO_2 in the gas phase. Crabtree himself has used Ringer bicarbonate and found the effec known by his own name. However, he only measured total gas exchanges over a range of 60 minutes but did not follow their time course. Warburg et al.[6][18] also, by means of their 'indirect method', measured the metabolic rate of carbohydrate in tumor tissues but again gave the overall values alone over a shorter range of 15 or 30 minutes. The same is the case with the results presented by Dickens and Simer[19] and by Elliott and Backer[20]. Thus, the time course of the Crabtree effet in the presence of bicarbonate seems in fact to have escaped attention.

As shown above, however, when bicarbonate is present the respiratory inhibition by glucose added does not sustain itself linearly as in Krebs-Ringer phosphate but displays a characteristic pattern. Namely, the initial drop of respiration is quite distinct but it begins to be restored at about 30 to 40 minutes of incubation period and follows increasing rates even to exceed the endogenous level of respiration. In the presence of bicarbonate therefore percentage inhibition in the Crabtree effect is to be dependent on the length of incubation period which was employed. The original data of Crabtree[2] obtained by 60 minutes' incubation show merely a weak inhibitory effect as low as 13%, and Elliott and Baker[20] who confirmed the Crabtree effect, employing an incubation period of 90 minutes, give also about 9% inhibition on an average. Such lower values of inhibition as compared with those obtained in Krebs-Ringer phosphate which are usually of the order of 30 to 40% seem likely to have resulted partly from admixture of lowered and raised phases of the Crabtree effect due to their long-term observation. In Krebs-Ringer phosphate, as well known, the lowered rate of glucose oxidation is sustained linearly. Inferentially, this would point to the inhibitory action of phosphate on the raised phase of the Crabtree effect.

It has already been recommended by Warburg et al.[4] that for the study of the real metabolic behavior of ascites tumor cells the homologous ascitic fluid fortified with glucose and bicarbonate is to be used and it is also reported by Fraser[21] that the presence of bicarbonate is of importance for promoting the incorporation in vitro of C^{14}-glycine into ascites tumor cell proteins. Recently, Seelich and Letnansky[22] followed the time course of the Crabtree effect in the presence of bicarbonate over a range of 90 minutes and demonstrated a linear pattern quite similar to that in Krebs-Ringer phosphate. However, it seems difficult to compare directly their findings with those in the present investigation in view of difference between experimental conditions employed, since Seelich and Letnansky have measured the O_2 uptake alone according to the Pardee methcd[5] using diethanolamine as a 'CO_2-buffur' and air containing 1% CO_2 as the gas phase.

On the other hand, it must also be added that Danes and Kieler[23], in agreement

with the present findings, has found the two-phase pattern of the Crabtree effect which was obtained by the Cartesian diver technique and with Yoshida ascites tumor cells when CO_2 and bicarbonate were present, even if their explanation of the CO_2 effect must be still open to question.

Respiratory quotient. The R.Q. of tumor tissues or cells has been measured by many workers, giving somewhat divergent values depending upon the methods employed and the kind of tumors studied. According to the classical data of Dickens and Simer[19)24)] which were obtained with solid tumors in the presence of glucose and with a experimental period of 5 hours, the R.Q. values are distinctly less than unity (0.8-0.9) irrespective of whether bicaronate is present or not. Recently, however, it seems to have been accepted that ascites tumor cells at least can oxidize fatty acids and predominant substrate in endogenous respiration is fatty matters.[17)25)26)27)28)] This is supported also by the endogenous R.Q. values (Table 1) obtained in the present investigation.

It has first been noted by Elliott and Baker[20)] that with Phila. No. 1 tumor the addition of glucose does cause a rise in R.Q. It has been reported by a number of workers[16)28)29)] that this shift of R.Q. is much more marked in ascites tumor cells, giving a value greater than unity at high glucose concentrations. It is interesting to note that the R.Q. values calculated from the data of Medes and Weinhouse[15)] where CO_2 output is measured as $BaCO_3$ for isotopic study are always greater than unity (1.1-1.3), provided that the concentration of glucose or fructose is 0.01M or above, whereas when lactate or acetate is added the R.Q. shift does not exceed unity. This rise beyond unity of R.Q. which was caused by glucose was compatible again with the findings of the present investigation (Table 1). Many experiments which will be shown in the succeeding report have given the same result. Moreover, it must be mentioned that the increased level of R.Q. is sustained throughout the experimental period of 120 minutes so long as glucose is present. From this, it is reasonable to say that not only there is an intimate relation between the presence of glucose and the rise of R.Q., but as pointed out by Weinhouse[23)], there occur reductive or synthetic processes with which the Crabtree effect of characteristic pattern is coupled in a definite manner.

In view of the facts that R.Q. of endogenous respiration is of the order of 0.7 which shifts beyond unity by the addition of glucose, that ascites tnmor cells can oxidize incorporated palmitate-1-C^{14}[27)], and that besides TPNH and ATP, bicarbonate is a limiting factor in the immediatesynthes is of fatty acids from acetyl CoA[30)31)], it is highly possible, as also suggested by Seelich[22)], that a synthetic process, if any, which is coupled with the Crabtree effect is to be predominantly fat synthesis from glucose.

It has been shown by Medes and Weinhouse[15)] that endogenous fat oxidation can be

substituted up to 70% by glucose oxidation when it is added. If this means a mere substitution between the two metabolites to be oxidized, the resulting R.Q. cannot surpass unity. As pointed out above, however, the R.Q. values computed from the data given by them are always more than unity when longlived Crabtree effect was occurring. This incompatibility may be indicative of the fact that the Crabtree effect does not consist in a mere substitution of glucose oxidation for fat oxidation but is a half manifestation of a highly integrated process of oxidation and synthesis which is peculair to tumor cells. Although it is the subject of further research to obtain a convincing picture of the Crabtree effect, it must be recognized at the present stage of investigation that the presence of bicarbonate is a limiting factor in an organized procession of chemical events under this effect, causing a marked change in the respiratory pattern and yet sustaining a R.Q. value greater than unity.

Summary

1) The Crabtree effect occurs independently of the concentration of glucose added and lasts so long as the glucose remains. Lactate or pyruvate added together with glucose can not remove the Crabtree effect, showing the dominancy of glucose.

2) When homologous ascitic fluid fortified after Warburg with bicarbonate was employed as suspension medium, the overall gas exchange of tumor cells shows a linear increase of positive or negative pressure according to the presence or absence of glucose, respectively. When glucose concentrations are low ($3 \cdot 10^{-3}$M and 10^{-3}M), linearly increasing positive pressure turns abruptly to negative pressure at certain points of incubation period and follows a linear line of negative pressure parallel to that in endogenous metabolism. This turning point of pressure is reached the sooner, the lower is glucose concentration.

3) Krebs-Ringer bicarbonate solution can replace completely the fortified ascitic fluid in respect to overall gas exchange.

4) The pattern of the Crabtree effect is greatly modified by the presence of bicarbonate and CO_2 in gas phase, namely, a marked fall of respiration at the early phase of incubation is followed by a rise of respiration which proceeds beyond the endogenous level.

5) The R.Q. value of endogenous respiration is of the order of 0.7, showing that endogenous metabolites consist chiefly of fatty substances. By contrast, the addition of glucose at high concentrations does cause a marked rise in R.Q., giving always a value greater than unity.

6) Resting upon these facts, it has been discussed that the Crabtree effect is a half manifestation of an integrated process of oxidation and reduction which is peculair to tumor cells and to which the presence of bicarbonate is of primary importance.

It is a pleasure to express our appreciation of the interest in the study shown by Dr. W. Nakahara. This study was aided in part by a Grant for the Scientific Research from the Ministry of Education given to the Research Group on the "Biochemical Study of Cancer".

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[GANN, 52, 293~303; December, 1961]

ON THE HISTOLOGICAL VARIATION OF LUNG CANCER WITH SPECIAL REFERENCE TO AGE FACTOR

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In continuation of our investigation on the variation of cancer hitology[6,20,21,22,23,24,26,27,28,29,30,33], we have conducted comparative studies between surgical and autopsy cases on the histological picture of lung cancer in relation to the age of the host, and some interesting results obtained will be presented and discussed here.

Material and Methods

Two hundred and twenty cases of primary lung cancer, 94 surgical and 126 autopsy cases, were employed. These cancer patients were from 27 to 80 years old and 184 of them were males and 36 were females.

The surgical cases were made available for examination by the favour of Dr. H. Ishikawa, Surgical Clinic of Keio University, Dr. H. Katsuki, Institute of Lung Cancer of Chiba University, etc. and some of autopsy cases were examined by the favours of Prof. K. Kageyama, Department of Pathology, Keio University, Prof. N. Takizawa, Dr. G. Ide, Department of Pathology, Chiba Univerity, Prof. T. Miyaji, Department of Pathology, Osaka University, and many other pathologists in Japan.

All these cancer tissues were fixed in formalin as usual and many paraffin sections were made from the primary and metastatic foci, and stained mainly with hematoxylin and eosin, but when necessary the silver impregnation of Bielshowsky, periodic acid-Schiff stain after Lillie, mucicarmine stain and v.Gieson and Weigert staines for elastic fibers were employed.

The histological pictures of the cancer tissue were classified according to the author's methods[30]. Few cancer parenchyma were seen in unaltered pulmonary alveolus, perivascular space or in mucinous secretion, but the majority of them were accompanied by interstitia classified into 4 types, namely, fibrillar, loosely fibrous, fibrous, and hyaline.

The histological appearances of cancer parenchyma and interstitia of many areas of the cancer tissues were compared with each other. And ranges and frequencies of appearances of various histological pictures were compared between the surgical and autopsy cases and according to age of the host.

In this study statistical observations were mainly made in male cases because of the smaller number of female cases.

Results

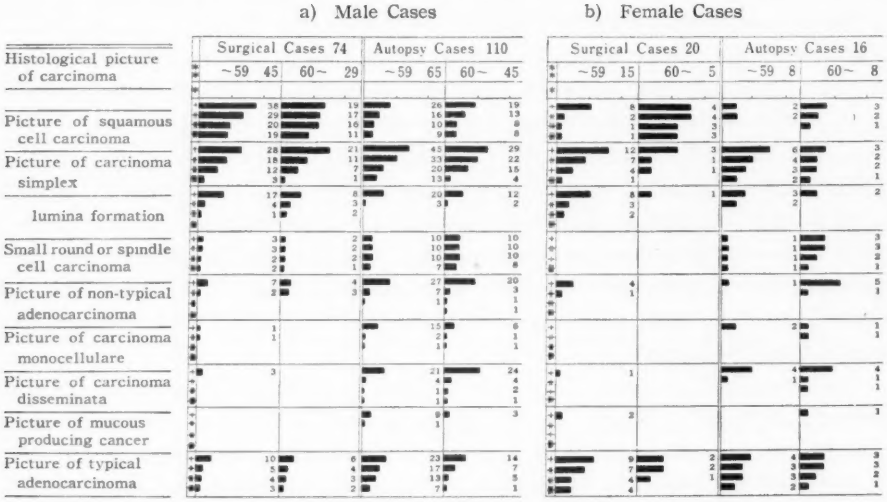
Results obtained are summarized and presented in Fig. 1 a), b), Fig. 2 a), b), Fig. 3 a,) b) and Table 1.

Histological picture of our lung cancer cases was generally very varied and even in the same specimen of the cancer tissue there were sometimes found many types of cancer histology, but among various types of cancer histology seen in the same case there were often noticed transitional pictures from one to the other.

As shown in Fig. 1 and Fig. 2, cancer cases with relatively simple histology were found more frequently in surgical cases and cases with more complicated histology were noticed in autopsy cases.

1) **Squamous cell carcinoma.** Generally speaking, the picture of squamous cell carcinoma was far more frequently seen in surgical cases than in autopsy ones, and cases with relatively simple picture of squamous cell carcinoma were also far more frequent in surgical specimens than in autopsy materials.

These tendencies were more remarkably noticed in younger age than in older one.



(Number of respective cases are indicated as percentage of numbers of cases in each group)

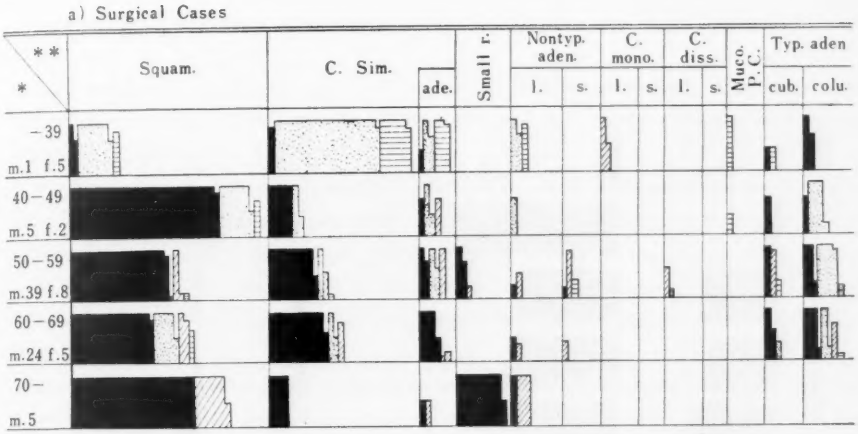
- \+ Cases with picture of respective cancer histology.
- ⫽ Cases with picture of respective cancer histology extending over more than 1/4 of the cancer mass.
- ⫽⫽ Cases with picture of respective cancer histology extending over more than 1/2 of the cancer mass.
- ⫽⫽⫽ Cases with picture of respective cancer histology extending over more than 3/4 of the cancer mass.

Numbers of respective cases are indicated in each space.

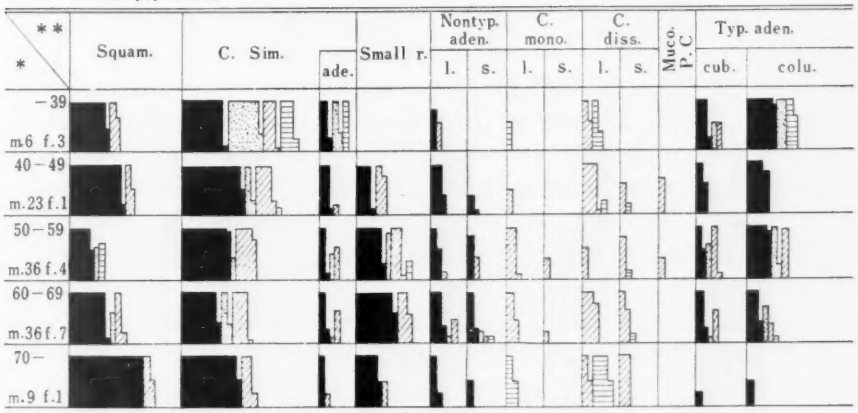
- ~59=Cases of age under 59 years,
- 60~=Cases of age above 60 years.

Fig. 1. Comparisons of range and frequency of appearance of various cancer histology in each age group according to sex.

a) Surgical Cases



b) Autopsy Cases



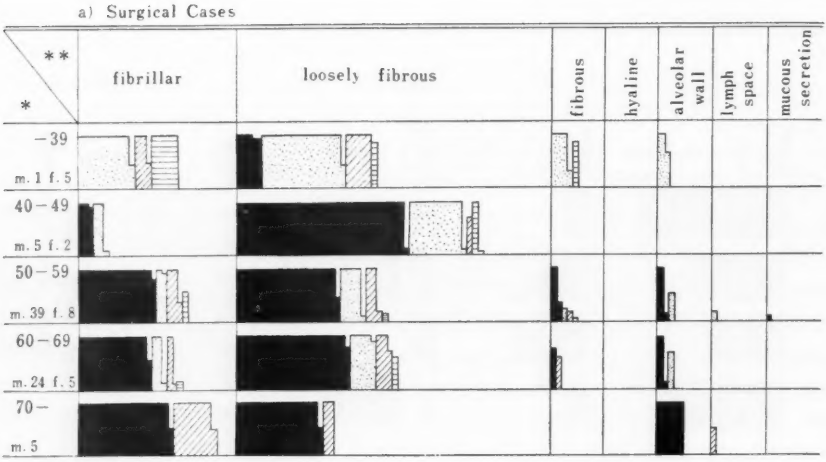
* Age (Number of male or female cases are indicated respectively in m. No. or f. No.)
-39=Cases of age under 39 years.
40-49=Cases of age from 40 to 49 years.
50-59=Cases of age from 50 to 59 years.
60-69=Cases of age from 60 to 69 years.
70- =Cases of age above 70 years.

** Histological picture of cancer parenchym.
Abbreviations:
Squam.=Squamous cell carcinoma, C. Sim.=Carcinoma simplex, ade.=Carcinoma simplex with lumina formation, l.=larger cancer cells, s.=smaller cancer cells, Small r.=Small round or spindle cell carcinoma, Non-typ. aden.=Non-typical adenocarcinoma, C. mono.=Carcinoma monocellulare, C. diss.=Carcinoma disseminata, Muco. P. C.=Mucous producing cancer, Typ. aden.=Typical adenocarcinoma, cub.=cuboidal cell, colu.=columnar cell.

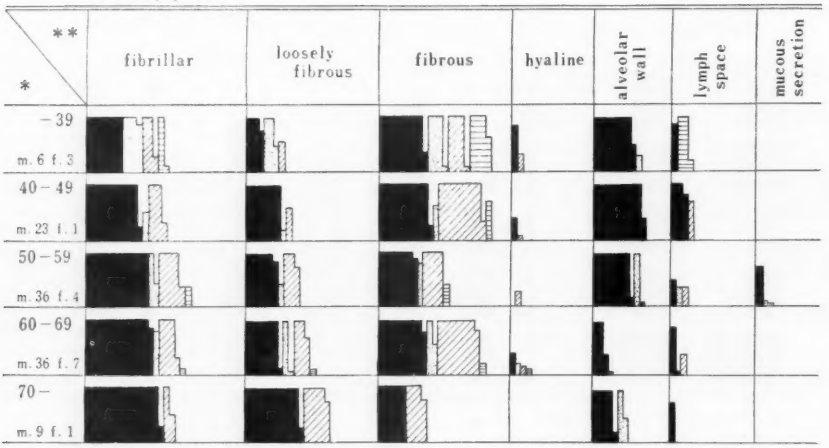
Width of histological areas with each histological picture of larger cancer alveoli in male cases.
Width of histological areas with each histological picture of larger cancer alveoli in female cases.
Width of histological areas with each histological picture of smaller cancer alveoli in male cases.
Width of histological areas with each histological picture of smaller cancer alveoli in female cases.

Fig. 2. Frequency of appearance of histological picture of cancer parenchym in each decade in surgical (a) and autopsy (b) cases.

a) Surgical Cases



b) Autopsy Cases



* Age (Number of male or female cases are indicated respectively in m. No. or f. No.)
-39=Cases of age under 39 years.
40-49=Cases of age from 40 to 49 years.
50-59=Cases of age from 50 to 59 years.
60-69=Cases of age from 60 to 69 years.
70- =Cases of age above 70 years.

** Histological picture of cancer stroma.

Width of histological areas with each histological picture of larger cancer alveoli in male cases.
Width of histological areas with each histological picture of larger cancer alveoli in female cases.
Width of histological areas with each histological picture of smaller cancer alveoli in male cases.
Width of histological areas with each histological picture of smaller cancer alveoli in female cases.

Fig. 3. Frequency of appearance of histological picture of cancer stroma in each decade in surgical (a) and autopsy (b) cases.

In surgical cases a picture of this cancer histology was more frequently seen in younger age. However, in autopsy cases this picture was more frequently seen in older age.

The interstitia of this cancer histology were fibrillar or loosely fibrous in almost all surgical cases. However, in autopsy cases, they were sometimes fibrous especially in younger age.

2) **Cacinoma simplex.** Generally, a picture of carcinoma simplex was seen somewhat more frequently in autopsy cases than in surgical cases, and this tendency was somewhat remarkably noticed in younger age.

In surgical cases, the interstitia of this cancer histology were generally loosely fibrous or fibrillar, but in autopsy cases they were more frequently fibrous especially in younger age.

3) **Small round or spindle cell carcinoma.** In surgical case, a picture of small round or spindle cell carcinoma was seen only in few cases However, in autopsy case, this picture was more frequently seen. And in almost all autopsy cases this histological picture was rather simple.

In surgical cases the interstitia of this histological type were fibrillar and sometimes loosely fibrous, but in autopsy cases they were sometimes fibrous especially in younger age.

4) **Carcinoma disseminata and carcinoma monocellulare.** Pictures of these cancer histologies were seen rarely and in narrow range in surgical cases, but somewhat frequently in autopsy ones. The picture of carcinoma disseminata made of smaller cancer cells was more frequently seen in older age.

The interstitia of carcinoma disseminata were generally fibrous, but in older age rather loosely fibrous.

5) **Non-typical adenocarcinoma.** The picture of non-typical adenocarcinoma was seen more frequently and in wider range in autopsy cases than in surgical ones. In autopsy case the cancer cells of this carcinoma type were more often smaller in size in older age.

The interstitia of this cancer histology were generally fibrillar in surgical cases, but sometimes fibrous in autopsy cases, being not influenced by age factor.

6) **Mucous producing cancer.** This picture was rarely seen in surgical cases, but more frequently in autospy cases, and rarely recognized in older age. No differences was noticed in character of interstitia of this cancer histology according to age.

7) **Typical adenocarcinoma.** On the picture of typical adenocarcinoma, in female cases, any no marked differences in appearance of this cancer histology could be found between autopsy and surgical cases, and in male cases, this cancer histology was somewhat frequently seen in younger cases than in older ones and somewhat more often in autopsy cases than in surgical ones.

In surgical cases, the interstitia of this cancer histology were generally loosely fibrous or fibrillar and rarely fibrous. However, in autopsy cases, they were more fibrous especially in younger age, and it was noticeable that in latter cases cancer cells of this cancer histology proliferated frequently into pulmonary alveolus.

8) **Histological pictures of cancer tissues in the cases where both surgical and autopsy specimens were examined.** Case 1, 30 year old male: Autopsy was performed 6 months after operation. Primary focus was supposed to be in the segmental bronchus of the right upper lobe. In surgical specimen there were seen pictures of squamous cell carcinoma, carcinoma monocellulare and of typical adenocarcinoma in almost same range and a picture of carcinoma simplex in a few areas. However, in autopsy material a picture of typical adenocarcinoma could not be noticed, and the major part of the tumor tissue showed a sarcoma-like picture of carcinoma disseminata, and in some areas there was seen a picture of non-typical adenocarcinoma made of larger and smaller cancer cells, in a few areas a picture of carcinoma simplex and only in a few areas a picture of squamous cell carcinoma. There could be recognized in some areas transitional pictures from one to the other. Interstitia were generally loosely fibrous and fibrillar and showed fibrous character only in some areas of autopsy specimen.

Case 2, 56 year old male: Autopsy was performed 1 year and 2 months after operation. Primary focus was supposed to be in the segmental bronchus of the left upper lobe. In surgical specimen, there was seen a picture of typical adenocarcinoma in almost the entire area. However, in autopsy material there could be seen pictures of squamous cell carcinoma and of carcinoma simplex besides a picture of typical adenocarcinoma, with transitional pictures in some areas.

Case 3, 63 year old male: Autopsy was performed 6 months after operation. Primary focus was supposed to be in the subsegmental bronchus of the right upper lobe. In surgical specimen, the major part of the tumor tissue showed a picture of carcinoma simplex and the minor part that of squamous cell carcinoma. However, the autopsy specimen showed mostly a picture of squamous cell carcinoma, with other areas showing pictures of carcinoma simplex, carcinoma disseminata and non-typical adenocarcinoma ; also transitional pictures in some areas. Interstitia of cancer tissue were generally loosely fibrous and fibrillar and showed fibrous character only in some areas.

9) **Correlation between the site of cancer origin and cancer histology in surgical and autopsy cases.** It is difficult to determine exactly the site of origin of lung cancer, it being possible to suppose by the site of cancer tissue and destructive pattern of the bronchus as indicated in Table 1. In this table, classification of lung cancer cases were made by the dominating histological picture of the cancer tissue. As shown in this table, our cases showed no special differences in distribution of site

of cancer origin between surgical and autopsy cases. In general, squamous cell carcinoma cases were far more frequently seen in surgical materials and carcinoma simplex and small round or spindle cell carcinoma cases, especially the latter, were far more frequent in autopsy cases. And in case of cancer of peripheral origin, it

Table 1. Correlation between histological classification and site of origin

	Surgical Case												Autopsy Case											
Site of Origin / Histological Classification**	Main Bronchus		Bronchial Truncus		Segmental Bronchus		Subsegmental Bronchus		Uncertain		Total		Main Bronchus		Bronchial Truncus		Segmental Bronchus		Subsegmental Bronchus		Uncertain		Total	
	m.	f.	m.	f.	m.	f.	m.	f.	m.	f.	m.	f.	m.	f.	m.	f.	m.	f.	m.	f.	m.	f.	m.	f.
Squamous Cell Carcinoma	3		2		26	2	6	2	4		41	4	5		3		10	2			3		21	2
Carcinoma Simplex	1	1	2		13	6	4	1	2	2	22	10	3		4		20	2	2	1	13	2	42	5
Small Round or Spindle Cell Carcinoma					3				1		4	0	3		2	1	7		2		6	2	20	3
Carcinoma Disseminata											0	0					2	1			1		3	1
Carcinoma Monocellulare											0	0					2						2	0
Non-Typical Adenocarcinoma											0	0							1		1		2	0
Typical Adenocarcinoma					6	5	1			1	7	6	1			1	7	3	8		5		20	5
Total	4	1	4	0	48	13	11	3	7	3	74	20	11	1	9	2	48	8	13	1	29	4	110	16

* Site of origin was supposed by the site of cancer tissue and destructive pattern of the bronchus.

** Histological classification was tentatively made by the histological picture seen in widest range of the tumor mass.

Numbers of male or female cases are indicated respectively in m. No. or f. No..

was especially noticeable that the pattern of appearance of squamous cell carcinoma and of typical adenocarcinoma was markedly different between surgical and autopsy cases; No special difference in the age factor was recognized between surgical and autopsy cases, excepting few cases of peripheral origin where the ages of autopsy cases were younger than surgical cases.

Summary and Discussion

Tauchi *et al*[30,33] and Sato[22] investigated the histological picture of cancer tissue examining the difference between autopsy and surgical cases, and presented some interesting conclusions as follows: The histological picture of cancer tissue was

generally relatively simple in the earlier stage of the disease but it became varied in the later stage, because various kinds of pictures were derived from the original cancer histology in the course of its growth. And they emphasized that influence of tissue environment may play an important part in the variation of cancer histology.

Then, Tauchi *et al*[23)33)] investigated the histology of gastric cancer and noticed some interesting relationship between variation of cancer histology in the course of growth or characteristics of their interstitia and age of the host.

The present authors have studied in the same way the histology of much larger number of cases of lung cancer and they found, as above mentioned, some interesting differences of histological picture of the cancer between surgical and autopsy cases; the former being generally considered to represent earlier stage of the disease and the latter cases later stage.

Histological pictures of the lung cancer might be considered to differ between surgical and autopsy cases because of different site of cancer origin of the material examined, influence of therapy such as radiation or drugs, and because of the age factor of the cases examined. However, between oursurgical and autopsy cases, there was recognized no special difference in the site of cancer origin, influence of cancer therapy and age factor, excepting few cases of cancer of peripheral origin. And so, the difference of histological picture between our surgical and autopsy materials may be due only to the difference of the stage of cancer growth, with few exceptions.

Varied histological picture of lung cancer was noticed by many authors,[3)7)11)12)13)14)16)17)26)34)35)], but according to our examination, the histological picture of this cancer was relatively simple in surgical materials and more varied in autopsy materials excepting in case of small round or spindle cell carcinoma.

In other words, it is considered that, as in other cancer cases examined by us, the histological picture of the lung cancer generally became varied in the course of cancer growth. This tendency was ascertained in cases where histological pictures could be examined both in surgical and autopsy materials in the same case.

In our cases examined, there were recognized transitional pictures among all histological pictures of cancer, and so, variation of histological picture during the course of cancer growth is considered to be derived from cancer histology existed in earlier stage. From the results obtained, it is also considered that the picture of the squamous cell carcinoma generally decreased in frequency during the course of cancer growth and this tendency was seen very markedly in younger age but not so markedly in older age.

However, a picture of carcinoma simplex had a tendency to become increased in autopsy cases especially of younger age, from which it might be considered that in the course of cancer growth the cancer cells become more undifferentiated in younger age than in older age.

The picture of small round or spindle cell carcinoma was rarely seen in surgical case, but frequently seen in autopsy case. On the frequency of appearance of this cancer histology there are many reports such as of Olcott (18%)[16], Bryson (36%)[1], Fischer (30%)[3], Reingold (25%)[19], and Tauchi (21%)[26], in autopsy materials, and of Gledhill (8%)[4], Gordon (15%)[5], and Moore (11%)[15], in case of surgical materials. Moersch and McDonald[14] stated that the surgical removal was the most difficult in case of small round cell carcinoma among the cases of bronchial carcinoma, and they emphasized the fact that the average age of these cancer cases was the youngest among the cases of the bronchial carcinoma. From the above mentioned findings, however, it may be reasonably considered that some of this picture of small round or spindle cell cancer was derived during the course of cancer growth from other histological pictures of cancer existed in earlier stage. And the autopsy cases with this cancer picture showed relatively simple histological picture of this cancer histology. From these facts, it is considered that this cancer histology might be of irreversible character, being induced by some environmental factors.

Some interesting results have been obtained on the picture of typical adenocarcinoma, but discussions on it including a picture of so-called bronchiolar carcinoma will be made in future.

The present authors also pointed out that in almost all cases of surgical removal the interstitia of lung cancer tissue were fibrillar or loosely fibrous and that in autopsy cases they were also frequently fibrillar or loosely fibrous in older age but more often fibrous in younger age. This suggests that in the course of cancer growth the interstitia proliferate more vigorously in younger age than in older age.

The present authors also have some interesting unpublished data of the experimental research of subcutaneous transplantation of Ehrlich's ascites tumor to the SIV mice of different age groups, showing that histological picture of transplanted tumor became generally varied in the course of cancer growth. And in older group of mice, the tumor tissue grew far less vigorously and the connective tissue proliferated less actively, forming larger cancer alveoli.

Takeuchi[25], one of our coworkers, had found the interesting difference in the distribution of acid mucopolysaccharide in the ground substance of cancer stroma according to the pattern of growth of Brown-Pearce carcinoma of the rabbit, and emphasized that young connective tissue composed mainly of chondroitin sulfate was favourable to the growth of tumor cells.

Some authors suggested that the growth of cancer tissue may be inhibited by vigorous proliferation of the interstitium[8][9], while others considered that vigorously proliferating stromal connective tissue was favourable to the growth of tumor cells[18][25][36]. At any rate, the age difference in changing process of histological picture of cancer tissue may be influenced by the character of interstitia which become

characterized in the course of cancer growth by the age factor.

Some characteristics of lung cancer, such as differentiation of cancer cells and proliferation of interstitia are considered to be closely connected with each other, and it is obvious that the age factor may play an important part in the process.

Conclusions

Histological investigation were made on the lung cancer, comparing between surgical and autopsy cases with special reference to the age factor of the host.

The histological picture of lung cancer tissue was generally considered to become varied in the course of cancer growth. A picture of squamous cell carcinoma generally decreased in frequency during the course of cancer growth and this tendency was seen very markedly in younger age but not so markedly in older age. A picture of small round or spindle cell carcinoma was considered to increase in frequency during the course of cancer growth.

Interstitia of the cancer tissue proliferated more vigorously in younger age, and some interesting relationships between the cancer histology and interstitia were discussed.

The authors wish to express their thanks to Profs. N. Takizawa, H. Katsuki, and G. Ide, Chiba University, Profs. H, Ishikawa and K. Kageyama, Keio University, and Prof. T. Miyaji, Osaka University, for generous supply of materials.

This investigation was aided by the "Grant in Aid for Fundamental Scientific Research" of the Ministry of Education.

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[GANN, 52, 305~312; December, 1961]

HISTOCHEMICAL DISTRIBUTION OF ALKALINE AND ACID PHOSPHATASES, ESTERASE, β-GLYCOSIDASES AND PROTEIN-BOUND SULFHYDRYL GROUPS IN EXPERIMENTAL CARCINOGENESIS OF THE MOUSE SKIN

(Plates XXV—XXIX)

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Many investigators have reported histopathological studies on experimental skin carcinogenesis, and also, various histochemical observations on human neoplastic tissues have been published. However, histochemical detection of enzymes during experimental carcinogenesis has hardly been made.

The present study was undertaken to determine the presence of enzymes, such as alkaline phosphatase, acid phosphatase, esterase, β-glucuronidase, β-glucosidase and β-galactosidase, and protein-bound sulfhydryl and disulfide groups by histochemical technique in the process of experimental carcinogenesis induced by methylcholanthrene in mouse skin.

Materials and Methods

The skin of the back of one hundred mice were painted with 0.5% methylcholanthrene in acetone twice a week for 20 weeks. Six of the animals were killed every other weeks. Immediately after removal of the treated epidermis, fresh frozen sections of the thickness 10-20μ were made in the cryostat at −20°C and fixed in 10% neutral formalin for 10 minutes after drying at room temperature. These serial tissue sections were used for the demonstration of enzymes and protein-bound sulfhydryl and disulfide groups, and one of the sections was also stained with hematoxylin and eosin.

Alkaline and acid phosphatases were confirmed with an azo coupling method. The demonstration of alkaline phosphatase was carried out in the following manner: The fixed tissue sections were incubated in a solution containing 10 mg of α-naphthyl phosphate, 20 ml of M/10 Clark and Lub's buffer (pH 9.2), and 20 mg of tetrazotized diorthoanisidine (diazo blue B) at 20°C for 20 minutes. After incubation the sections were rinsed in distilled water, dehydrated and mounted in balsam. The positive reaction for enzyme activity was purple in a yellow or yellowish brown background.

As for acid phosphatase, the sections were incubated in a solution consisting of

10 mg of α-naphthyl phosphate, 20 mg of M/10 acetate buffer (pH 5.8), and 20 mg of diazo blue B at 20°C for 30 minutes. After incubation, the sections were treated in the same manner as above. The reactions were determined by the formation of a purple black and black azo dye.

In order to demonstrate nonspecific esterase, sections were treated with a solution prepared by mixing 10 mg of β-naphthyl acetate dissolved by 0.5 ml of acetone, 20 ml of M/10 Michaelis buffer at pH 7.8, and 20 mg of diazo blue B for 30 minutes at 20°C. The area with enzymatic activity showed a bluish purple color.

Histochemical technique for demonstration of β-glucuronidase, β-glucosidase, and β-galactosidase was carried out in the same manner except for using different substrates in accordance with the Seligman method. For the demonstration of β-glucuronidase, sections were incubated for 6 hours at 37°C in a solution prepared by mixing 7.5 mg of 6-bromo-2-naphthyl-β-D-glucuronide, 5 ml of methanol, 5 ml of phospho-citric buffer at pH 4.95, and 20 ml of distilled water. The sections were then washed briefly in water and treated with 20 ml of 0.02 M phosphate buffer (pH 7.5) containing 20 mg of diazo blue B in a refringator at 4°C. 6-Bromo-2-naphthyl-β-D-glucopyranoside was used as a substrate for β-glucosidase and 6-bromo-2-naphthyl-β-D-galactopyranoside for β-galactosidase. With these three glycosidases the staining reaction in the active sites were similar with a blue color.

Sulfhydryl and disulfide groups were demonstrated according to the Gomori method. Fresh frozen sections were fixed for 10 minutes in a 1% solution of trichloroacetic acid in 80% alcohol after drying at room temperature. Then the sections were rinsed in water and incubated for 8 hours at 37°C in a solution consiting of 10 ml of 10% KCN, 20 ml of 0.5 M borate buffer (pH 8.5-8.8), 25 mg of neotetrazolium chloride dissolved by 5 ml of absolute ethanol, and about 15 ml of 0.1 M acetic acid citrated with a drop of phenolphthalein. The mixture is completely decolored with 15 ml of 0.1 M acetic acid. The sections were washed in water, immersed in 1% acetic acid, and rinsed in water. In order to demonstrate sulfhydryl and disulfide groups together, reduction of disulfide groups is necessary. The reducing reagent was prepared by the addition of 100 ml of 0.4 M thioglycolic acid which was made to pH 8.0 with about 10 ml of 0.1 N NaOH. The sections were immersed for 2 hours at 50°C and rinsed in 1% acetic acid. The sulfhydryl and disulfide groups positive sites were stained a reddish purple.

Results

Normal skin. The alkaline phosphatase activity of normal skin was observed in the hair follicles and blood capillaries with no enzymatic activity in the epithelium. Acid phosphatase was distributed in the cornified and granular cell layers of the eithelium and hair follicles. Esterase was contained in the all layers except for the

superficial cell layer and hair follicles. The distribution of β-glucuronidase activity was rich in the granular, germinative layer and hair follicles, and the localization of β-glucosidase and β-galactosidase was similar to that of β-glucuronidase. Intense reaction of sulfhydryl and disulfide groups was found in the cornified and the granular layer and hair follicles.

3rd week. Epidermis and dermis became slightly hyperplastic and hair follicles were almost destroyed. Basal cells of the epidermis began to proliferate invasively to the subcutaneous connective tissue.

In this stage the activity of alkaline phosphatase was almost the same as that of normal skin, though its activity in hair follicles was slightly increased. Acid phosphatase showed a slight increase in the granular layer and a slight reaction was observed in the germinative layer. Fairly intense reaction of esterase was found in the granular layer. Its activity in other portions was nearly the same as that of normal skin. Protein-bound sulfhydryl and disulfide groups revealed similar distribution to normal skin.

8 th week. Treated epithelium exhibited more hyperplastic proliferation. Basal cells showed irregular arrangement with the basal membrane partly destroyed, and fibrous hyperplasia in the dermis was progressed.

In this stage intense activity of alkaline phosphatase was observed in the portion adjacent to proliferating epithelium. The localization of other hydrolytic enzymes and sulfhydryl and disulfide groups was the same as in the former stage.

12 th week. Hyperplasia of the treated epithelium was futher progressed. In some regions, cells became irregular in shape and size, and their nuclei showed pyknosis and karyolexis. Histological examination of several sections in this stage presented the picture of cancer.

The distribution and localization of alkaline phosphatese, acid phosphatase, esterase, and SH and SS-groups showed no change from these of the 8 th week.

16 th week. Many carcinoma pearls were found in tumor tissue and the tumor cells invaded into the subcutaneous connective tissue. Most of tumors were diagnosed as squamous cell carcinoma.

The alkaline phsophatase activity entirely disappeared. Acid phosphatase was not contained in tumor tissues except for surrounding tissues of horn pearls with a moderate reaction. Intense activity of esterase was observed in the cell layer corresponding to the granular layer of carcinoma pearls with a reddish purple color and several tumor tissues showed weak homogenous reaction in the stroma. β-Glucuronidase, β-glucosidase and β-galactosidase were abundantly contained in tumor tissues. The localization and distribution of SH-groups was similar to that of acid phosphatase. In the portion of pearl formation it exhibited moderate activity.

Discussion

Ih the present experiment the localization of alkaline phosphatase has shown most interesting findings. According to many investigators, the activity of alkaline phosphatase in intact skin was localized in blood capillaries and hair follicles, and not in epithelial cells. Kidd, Winzler and Burk[10] stated that its activity was very intense both in the stroma and in epidermal cells. Biesele and Biesele[5] reported that the enzyme in tumor tissues was contained copiously both in the stroma and in epidermal cells lying adjacent to the stroma. However, in the present study, alkaline phosphatase activity was never observed in tumor tissues and in epithelial cells throughout the experiment. Alkaline phosphatase was moderately localized in the connective tissue associated with the proliferating epithelium and in consequence of the progress of epithelial proliferation more intense reaction came to be observed. Though it was completely absent in cancerous tissues. Richardson and Pearson[17], describing their experimental mammary tumor in mice, stated that alkaline phosphatase activity was not dependent on the malignancy or anaplasia of the tumor, but on the activity exhibited by the luminal borders, myoepithelial cells, and endotheliallined spaces. According to Kabat and Furth,[11] a large amount of alkaline phosphatase was found in an osteogenic tumor of the mouse, and this enzyme was important in bone formation and might aid in the histological identification of osteogenic cells. Some experiments indicated that alkaline phosphatase was found in the fibroblastic regions of skin and in the chronic inflammatory tissues. From these studies, it is suggested that alkaline phosphatase seems to play some part in fibroblastic property in the proliferating malignant tumor.

Acid phosphatase in normal skin was found in whole epithelial cells and hair follicles, and its strong activity was observed in the cornified and the granular cell layer. Its reaction in the stages of carcinogenesis revealed the same localization as in intact skin. Lemon, Davison and Asimov[12] reported that acid phosphatase in human tissues was essentially unchanged from normal in benign tumors and inflammatory lesions. It was recognized by Reiner, Rutenburg and Seligman[16] that acid phosphatase in human normal epidermis showed considerable activity while basal cell carcinoma stained less intensely than even the basal layers of the homologous normal epidermis and some of the surface epithelium lost acid phosphatase activity. Acid phosphatase in ameloblastoma[15] was localized in the parenchyma of the tumor and cornified portions of it reacted intensely. The results of the present investigation coincided with these reports.

Esterase of normal skin was locaized in the germinative layer. In carcinomatous tissues, its activity revealed almost the same distribution and localization as that of normal skin. According to Cohen, Nachlas and Seligman,[6] the activity of esterase

of human tumors was low or frequently absent and did not correlate with the degree of differentiation of the tumor. On the other hand, Argyris[1),2)] concluded that intense activity in the mouse was observed in the epidermis and hair follicles during the resting phase of the hair cycle, and little activity of esterase was found in the regenerating or growing hair follicles and the hyperplastic epidermis during the growth phase of the cycle. The epidermis and hair follicles during wound healing in mice exhibited intense esterase activity. In ameloblastoma[18)], the distribution and localization of acid phosphatase and esterase showed similar patterns to each other.

The histochemical determination of β-glucuronidase has been established by two methods, one is the ferric-8-hydroxyquinolin method and other the post-azo coupling method using 6-bromo-2-naphthyl-β-D-glucuronide as the stubstrate. β-Glucuronidase localization with the post-azo coupling method in human tumors has been described by Monis, Banks and Rutenburg[13)]. They stated that human malignant epithelial tumors generally stained intensely and both in neoplastic and normal tissues its activity was observed in the cytoplasm. According to Fishman, Baker and Borges[7)], the localization of β-glucuronidase using the 8-hydroxyquinolin method was in the cytoplasm of neoplastic tissues, and fibroblasts and fibrocytes of non-neoplastic cells. In adamantinoma[15)], the activities of β-glucuronidase, β-glucosidase and β-galactosidase were mainly observed in the parenchyma of tumor tissues, and moderate or slight activity was found in proliferative or inflammatory connective tissues. Human carcinomas of the stomach, colon, breast and liver showed intense activity of β-glucuronidase, and sarcomas of mice induced by methylcholanthrene exhibited a largely negative reaction[14)]. It is noted that in demonstrating β-glucuronidase activity the genetic background of the animals is an important factor, and that sex hormones have the ability of increasing the β-glucuronidase activity of certain tissues in mice[8)]. In this study intense β-glucuronidase activity was found both in the germinative layer and neoplastic tissues. The localization and distribution of β-glucosidase and β-galactosidase exhibited similar distribution patterns in comparison with β-glucuronidase.

Sulfhydryl and disulfide groups in malignant neoplastic tissues have been confirmed by several workers. SH and SS in normal skin were rich in epithelial cells and hair follicles, particularly in the cornified and the granular layer similar to acid phosphatase. In cancerous tissues the granular, the cornified layer and horn pearls reacted intensely. Hayashi[9)] obtained almost the same results in his histochemical work. The SH reaction in malignant tumor tissues was also described by Bahr and Moberger[3)] to be more intense in tumor tissues than in normal tissues, and its activity was intense in the nuclei and especially the enlarged nucleoli in low differentiated carcinomas. In parakeratosis, the keratin is more strongly stained than the cellular layers and epidermis and the parakeratotic layers reacted strongly[19)].

Wingo and Foraker[20] reported that SH was found in all of the viable neoplastic cells and SS was contained in regions of intracellular keratinization and in epithelial pearls. Benign tumors seem to contain less SH than malignant ones[4].

Summary

A histochemical study under the condition of experimental skin carcinogenesis induced by 20-methylcholanthrene on the back of the mouse was carried out.

1. In processes of malignant growth, acid phosphatase, nonspecific esterase, β-glucuronidase, β-glucosidase and β-galactosidase showed nearly the same localization as in normal skin.

2. Protein-bound sulfhydryl and disulfide groups also exhibited similar patterns to intact skin.

3. Alkaline phosphatase gradually increased its activity in the portion adjacent to the proliferating epithelium but was completely inactive in cancerous tissues.

Acknowledgements

The authors wish to express their thanks to Prof. Kensaku Kawakatsu and Assist. Prof. Masahiko Mori, Second Department of Oral Surgery, Osaka University Dental School, for their advices in histochemistry.

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Explanation of Figures (Plates XXV—XXIV)

Figs. 1-6: Normal skin of the back. ×60.

1: Hematoxylin-Eosin (H-E)

2: Alkaline phosphatase (ALPase). Hair follicles and blood capillaries contain ALPase.

3: Acid phosphatase (ACPase). The cornified and granular layers and hair follicles have this enzyme activity.

4: Esterase: All cell layers of the epithelium except for the superficial layers and hair follicles display esterase activity.

5: β-Glucuronidase. The granular and germinative layers and hair follicles show its activity. Subcutaneous fatty tissues also reveal activity.

6: SH+SS: The cornified and granular layers and hair follicles react intensely.

Figs. 7-11: 3rd week after application of 20-methylcholanthrene ×60.

7: H-E. Epidermis and dermis becomes hyperplastic proliferation.

8: ALPase. Hair follicles show intense reaction.

9: ACPase. The granular layer exhibits more enzymatic activity.

10: Esterase. The superficial layers are rich in esterase.

11: SH+SS: Similar observation to Fig. 6 is noticed.

Figs. 12-15: 8th week after application. ×60.

12: H-E. More hyperplastic proliferation of the treated epithelium is observed.

13: ACPase. The cornified and granular layers reveal enzyme reaction.

14: Esterase. The superficial layers are stained moderately.

15: SH+SS: The superficial layers contain SH and SS.

Figs. 16-18: 12th week after application. ×400.

16: H-E: Skin cancer is produced in the treated area.

17: ALPase: Surrounding subcutaneous connective tissues of cancerous tissues contain ALPase.

18: Esterase. Almost the same view as Fig. 14.

Figs. 19–23: 16 th week after application. × 400

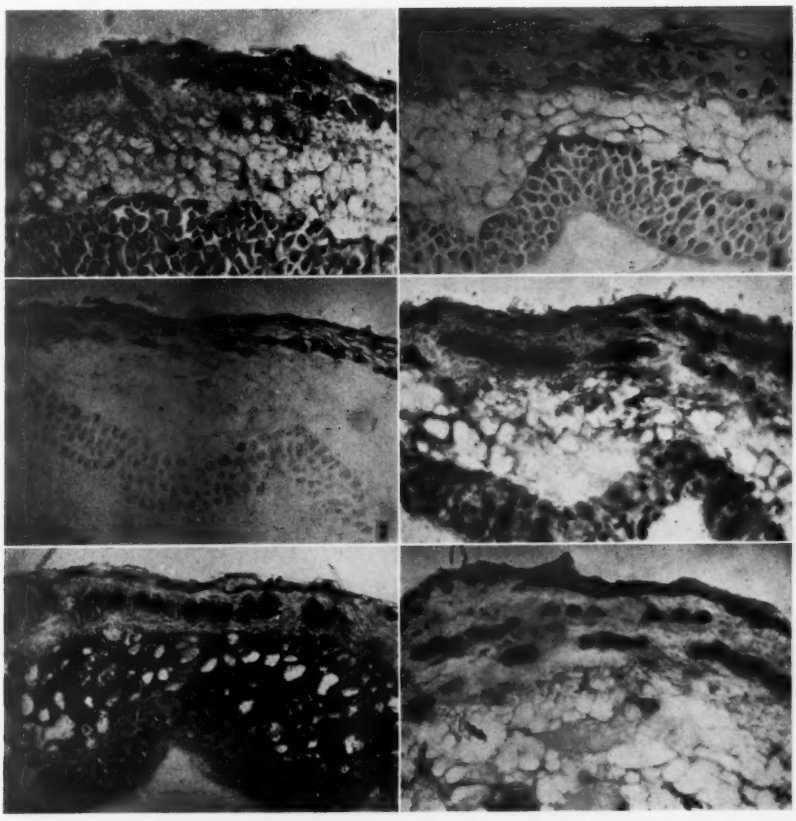
19: Esterase. Intense reaction is found in the granular layer.

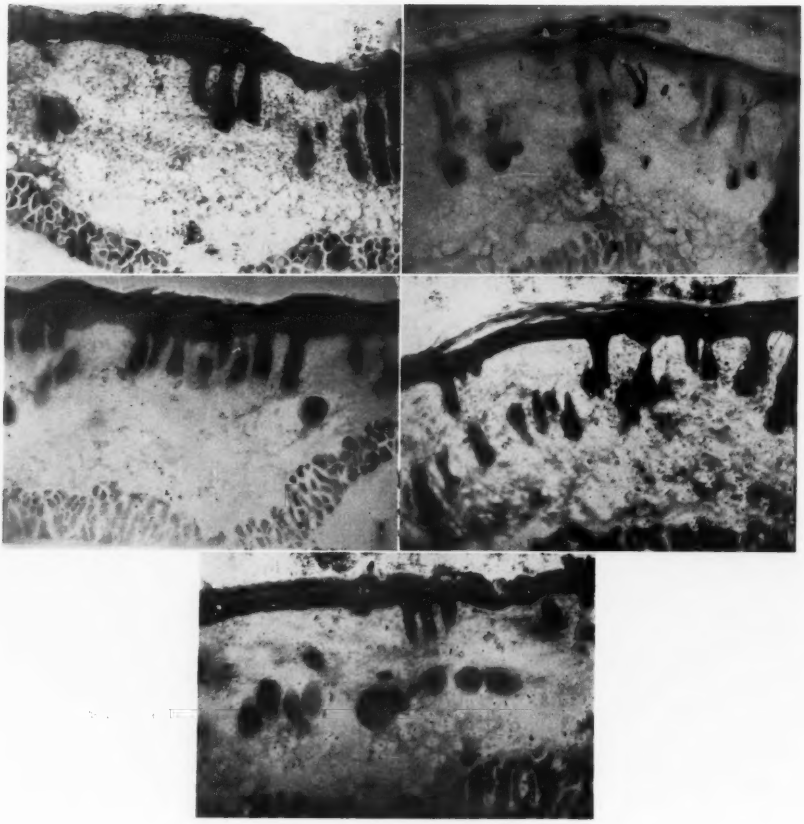
20: H-E: Many horn pearls are observed in cancerous tissues.

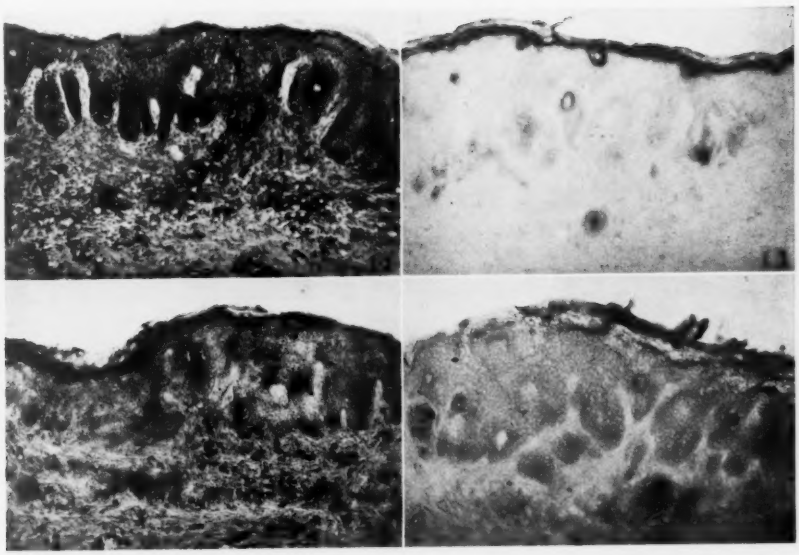
21: ACPase: No enzyme activity is observed in tumor tissues.

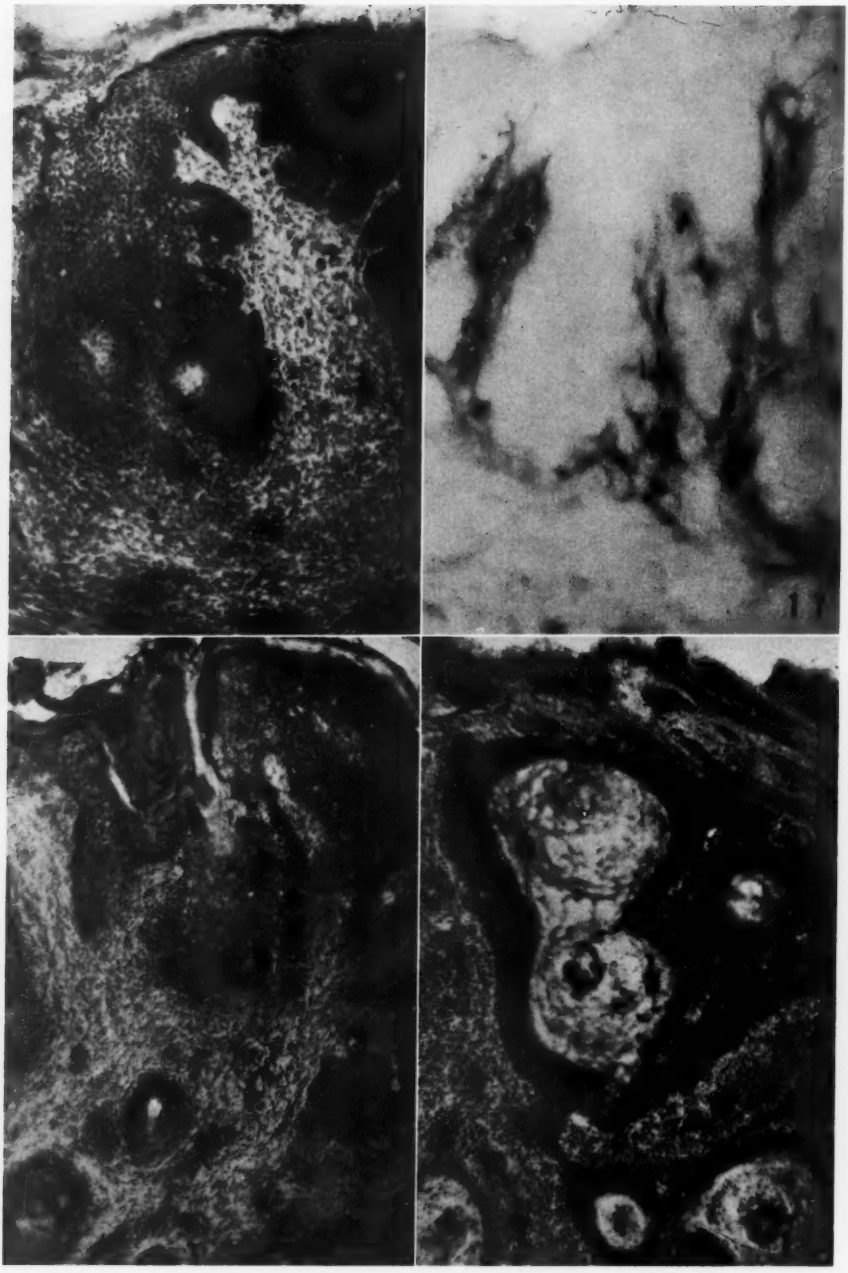
22: β-Glucuronidase: Strong activity is shown in tumor tissues.

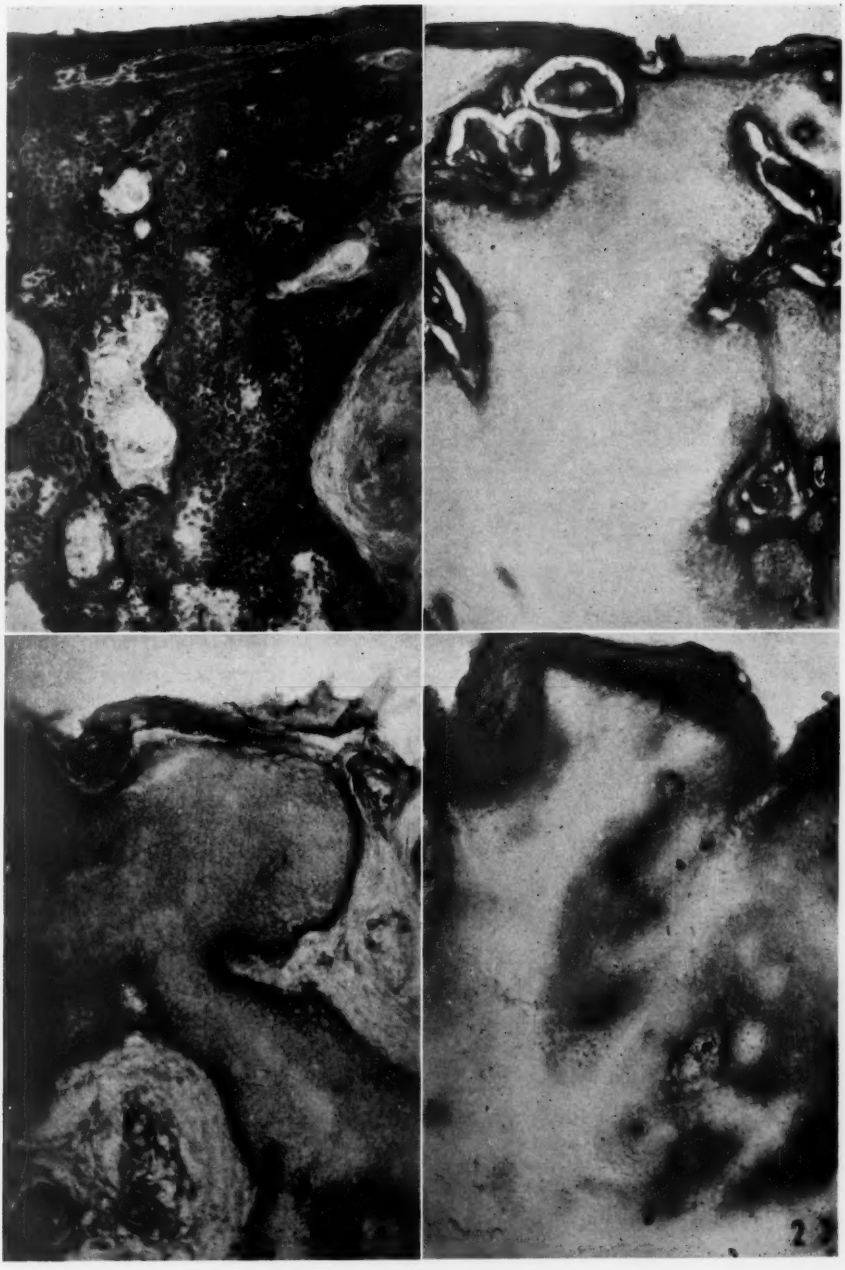
23: SH+SS: The cornified and granular layers exhibit intense reaction.











[GANN, 52, 313~320; December, 1961]

CHROMOSOMAL ALTERATION AND THE DEVELOPMENT OF TUMORS, VIII. AFFINITY OF DIPLOID AND POLYPOLID TUMOR CELLS OF COMMON YOSHIDA SARCOMA STRAINS TO CERTAIN ORGANS[1)2)]

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In Yoshida sarcoma which is characteriged by a subdiploid stemline, polyploid cells are usually observed in its cell population at a low percentage (Yosida 1948, 1952, 1960, Makino and Kanô 1951, Makinô and Yosida 1951, Makino 1952, 1957, Tjio and Levan 1956 and others). In the course of transplantation of Yoshida sarcoma the present author has found temporary proliferation of tetraploid cells after transplantation to a Buffalo strain rat (Yosida 1960). It was found in this tumor that cells with tetraploid karyotypes had a stronger affinity to the lung than those with diploid karyotype (Yosida and Tsuji 1960).

In order to investigate more exactly this problem, a common strain of Yoshida sarcoma which contained polyploid cells at a low percentage was used in the present study. The present report deals with a karyological study of tumor cells which infiltrated into spleen, kidney, thymus, lung and lymphnodes of tumor bearing rats.

Material and Methods

Yoshida sarcoma used in the present study was obtained from the Phermacological Research Institute, Tokyo, in December of 1959 and since that time it has been serially transplanted to various inbred strain rats kept in author's laboratory.

The Karyological and quantitative analysis of tumor cells which infiltrated into organs was carried out by the following technique. A part of organs of the tumor bearing rat was cut off and minced into small piecesss which were inoculated into the peritoneal cavities of new hosts by a transplantation needle. By this method the tumor cells which infiltrate into the organs can proliferate in the peritoneal cavity as a form of ascites tumor. The cell population in the ascites tumor thus developed represents an expansion of the infiltrated cell population. A practical technique of organ transplatation has been described in the previous papers of this series (Yosida

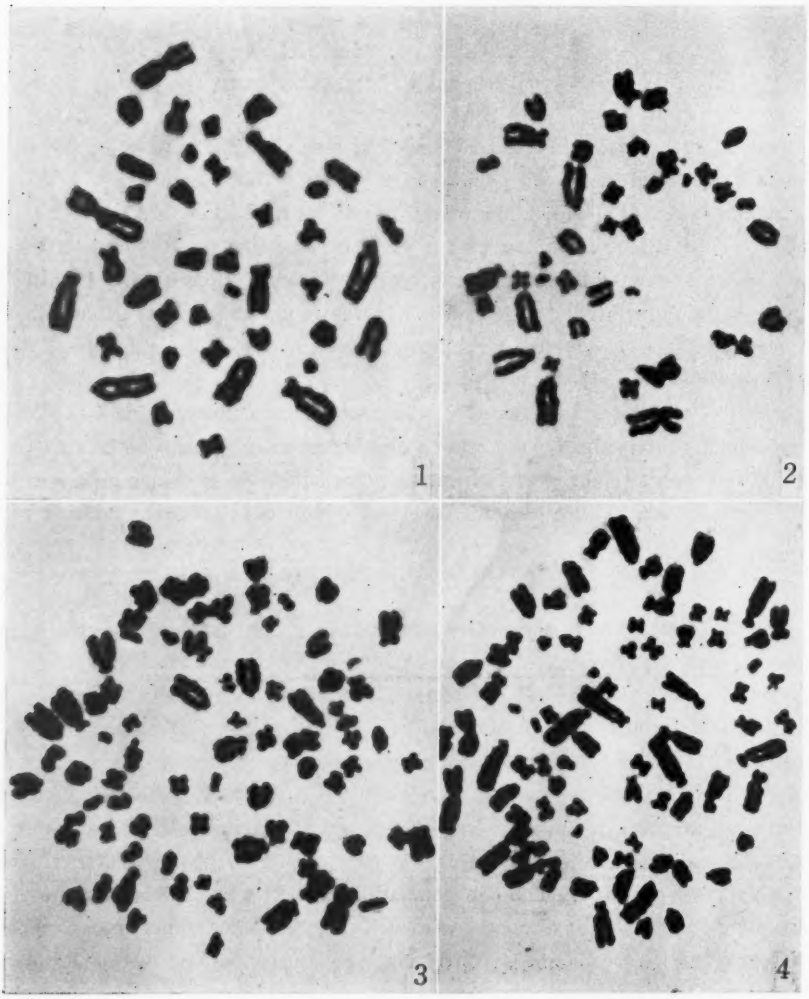
1) Contribution from the National Institute of Genetics, Japan, No. 395.

2) Supported by a Grant in Aid for Fundamental Scientific Research from the Ministry of Education (No. 0434 in 1961).

1959, Yosida and Tsuji 1960).

Tumor cells were observed by acetic dahlia spuash technique after pretreatment with hypotonic solution. About 100 to 200 tumor cells showing clear metaphase figures were sampled at random from three to four preparations, and the chromosomes were counted to determine the ploidy.

Results of Experiments



Figs. 1–4. Photomicrographs of Yoshida sarcoma cells with 40 chromosomes (Figs. 1 and 2), 79 chromosomes (Fig. 3) and 80 chromosomes (Fig. 4).

Experiment I. Tissue pulps of spreen, kidney, thymus and lung of a rat bearing a Yoshida sarcoma (# 105) were inoculated into the peritoneal cavities using for each of those organs four to five rats, respectively. Spleen transplantation to two rats, kidney inoculation to one and two thymus inoculations were successful and ascites tumors developed in their peritoneal cavities.

1. The frequency of diploid and polyploid tumor cells in the original Yoshida sarcoma (# 105): Original Yoshida sarcoma which was used in organ transplantation contained diploid tumor cells at 92.8% and tetraploid cells at 7.2% (Figs. 1-4). In the next transplant generation (# 110) by usual transfer of ascites tumor, the number of tetraploid cells was slightly reduced to 7.2%, whereas that of diploid cells increased to 93.0% (Table 1).

Table 1. Frequency of ploid cells in the original Yoshida sarcoma (#105) and in the 1 st transplant generation by usual transfer technique.

Ploidy / Transpl. gener.	±2n	±3n	±4n	±8n	No. of cells observed	Strain of rats
Original (# 105)	92.8%	0	7.2	0	69	Wistar
1 st (# 110)	93.0	0	7.2	0	125	〃

2. Ascites tumor developed by spleen inoculation: In two rats (# 127 and # 129) an ascites tumor developed by spleen inoculation. In one rat the number of polyploid cells in the ascites tumor (# 129) thus developed increased markedly to 34.1%, while diploid cells decreased to 65.9%. In the other rat (# 127) the number of polyploid cells increased slightly to 12.0%. There is a considerable difference between # 127 and # 129 rats regarding the frequency of polyploid cells. It may be due to the difference of animal strains used, because the former (# 127) was a Fischer-strain rat and the latter (# 129) a CW-strain rat. Ascites tumors in the # 129 rat were inoculated into the peritoneal cavities of two Buffalo strain rats (# 254 and 255) by usual transfer technique. Interesting is that the number of polyploid cells increased

Table 2. Frequency of ploid cells in ascites tumors developed by inoculation of spleen tissues of Yoshida sarcoma rat # 105

No. of tumor	Technique of transfer	Ploidy				No. of cells observed	Strain of rats
		±2n	±3n	±4n	±8n		
127	Spleen inocul. from # 105	88.0	2.6	9.4	0	92	Fischer
129	〃	65.9	0	33.3	0.8	126	CW-rat
254	Ascit. tum. inocul. from # 129	49.1	1.9	49.0	0	110	Buffalo
255	〃	44.7	0.8	51.5	2.0	132	〃

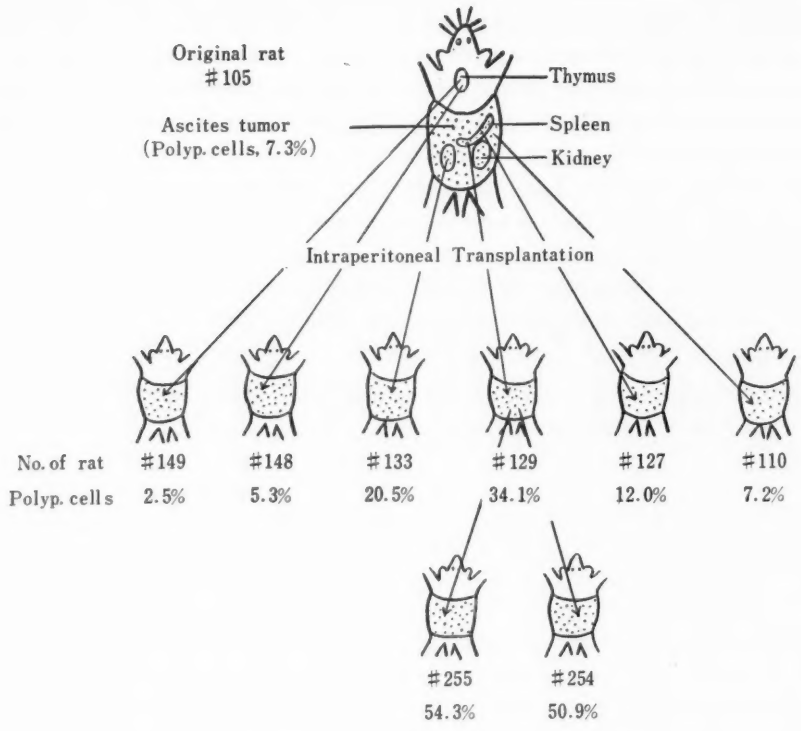


Fig. 5. Scheme of inoculations with some organs of rat # 105 bearing the Yoshida sarcoma.

to 50.9 and 54.3%, respectively (Table 2 and Fig. 5).

3. Ascites tumor developed by kidney transfer; Only one rat (# 133, CW-strain) developed the ascites tumor by kidney inoculation. In the cell population of the ascites tumor the number of diploid and polyploid cells was 79.5 and 20.5%, respectively. One rat (# 132, Long Evans strain) developed a solid type tumor in the peritoneal cavity by kidney inoculation. The solid tumor and the kidney of this rat were inoculated into three and four animals, respectively. Only one rat (# 200, Long Evans strain) by kidney inoculation developed as ascites tumor. In this ascites tumor, however, the polyploid cells did not increase as markedly as in the former case. They occurred only at 10.9% (Table 3 and Fig. 5).

4. Ascites tumor developed by thymus inoculation: Hypertrophic change of thymus was found in the rat # 105 bearing Yoshida sarcoma. It must have been caused by metastasis of tumor cells. Minced pieces of the thymus were inoculated into intraperitoneal cavities of two rats (# 148, CW-strain and # 149, WPY-strain). They

Table 3. Frequency of ploid cells in ascites tumors developed by inoculation of kidney tissues of Yoshida sarcoma rat # 105

No. of tumor	Technique of transfer	Ploidy				No. of cells observed	Strain of rats
		±2n	±3n	±4n	±8n		
133	Kidney inocul. from # 105	79.5	1.1	18.3	1.1	92	CW
200	Kidney inocul. from # 132*	90.0	0	10.0	0	105	CW

* A solid tumor developed in the intraperitoneal cavity of # 132 rat by transfer of kidney tissue of # 105 rat.

Table 4. Frequency of ploid cells in ascites tumors developed by inoculation of the thymus gland of Yoshida sarcoma rat # 105

No. of tumor	Ploidy				No. of cells observed	Strain of rats
	±2n	±3n	±4n	±8n		
148	94.7	0	5.3	0	19	CW
149	97.5	0	2.5	0	205	Wayne pink eyed

developed ascites tumors, but one (# 148) of them showed very poor growth. Although only 19 metaphasic cells were observed in the # 148 rat, 205 mitotic cells were observed in the other rat (# 149). It is quite interesting that the frequency of polyploid tumor cells in both rats # 148 and # 149 was reduced to 5.5 and 2.5%, respectively (Table 4 and Fig. 5). The latter figure is more reliable than the former, because many more cells in the latter case were analysed than in the former. This means that the affinity of diploid tumor cells to the thymus is stronger than that of polyploid cells.

Experiment II. In the first experiment, the ascites tumor by intraperitoneal inoculation of the lung tissue of a tumor-rat failed to develop. Therefore an experiment with lung tissue inoculation was carried out again using another usual strain (YB-9) of Yoshida sarcoma. This strain is a descendant of the Yoshida sarcoma

Table 5. Frequency of ploid cells in the original Yoshida sarcoma (YB-9) and in the 1st and 3rd transplant generation by usual transfer technique

Transpl. gener.	Ploidy				No. of cells observed	Strain of rat
	±2n	±3n	±4n	±8n		
Original (YB-9)	97.2	0.7	2.1	0	142	Wistar
1st (YB-20)	91.8	2.1	6.1	0	98	〃
3rd (YB-36)	91.8	1.8	6.4	0	109	〃

used in the control of the former experiment.

(1) Frequency of diploid and polyploid cells in the YB-9 Yoshida stock: Original Yoshida sarcoma YB-9 used in the present experiment showed the number of diploid cells at 97.2% and that of polyploid cells at 2.8%. In the next (YB-20) and the 3rd transplant generation (YB-36), however, the number of diploid tumor cells decreased to 91.8%, respectively (Table 5 and Fig. 6).

(2) Ascites tumor developed by lung inoculation: Minced lung tissue of the YB-9 rat were inoculated into 6 rats of the Long Evans strain. Among them 2 rats (YBL-3 and YBL-6) developed ascites tumors. One ascites tumor (YBL-3) was shown to have the number of diploid cells at 83.4% and of polyploid cells at 16.6%. A higher percentage of polyploid cells in ascites tumor was found in the YBL-6 rat. The frequencies of diploid and polyploid cells in this tumor were 68.6 and 31.4%, respectively (Table 6 and Fig. 6).

Table 6. Frequency of ploid cells in ascites tumors developed by inoculation of lung tissues of Yoshida sarcoma rat YB-9

No. of tumor	Ploidy				No. of cells observed	Strain of rats
	±2n	±3n	±4n	±8n		
YBL-3	83.4	0.4	16.2	0	235	Long Evans
YBL-6	68.6	1.5	29.9	0	167	〃

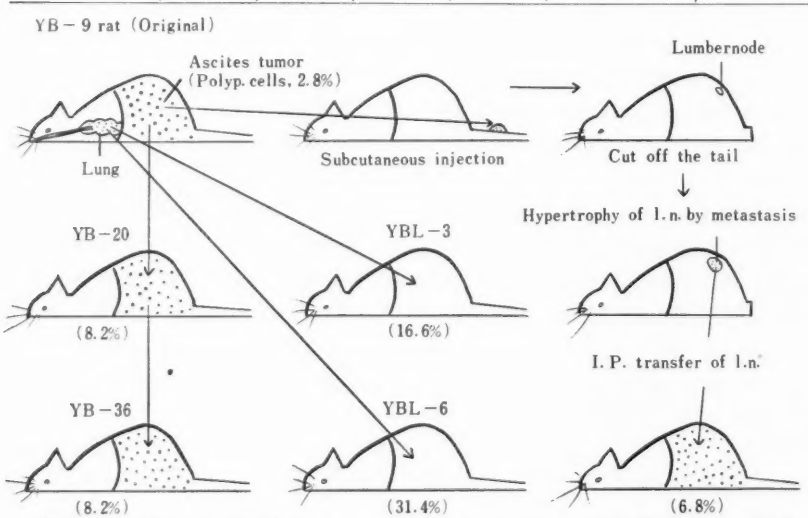


Fig. 6. Scheme of transfer of lung tissue of a tumor bearing rat (YB-9) and of hypertrophic lumbarnode developed by subcutaneous injection into the tail.

Experiment III. In experiment I it was found that diploid tumor cells have

stronger affinity to the thymus gland of the tumor bearing animals. In order to know whether the diploid tumor cells have also stronger affinity to other glands besides the thymus the following experiment was carried out using Yoshida sarcoma YB-9. According to Sato (1959), tumor cells of MH mouse ascites hepatomas inoculated under the tail skin of C3H mice infiltrated selectively into the lymphnodes. In order to obtain a metastasis of the Yoshida sarcoma cells into the lymphnodes, the ascites tumor of YB-9 rat was injected under the skin of the tails of three rats. After one week, the tails were cut off at the base. 22 days after the transplantation, these rats were opperated and the lymphnodes examined. Abnormal hypertrophy of the lumbar lymphnode was found in one rat. The lymphnode was minced into small piecess which were transferred into intraperitoneal cavities of two rats. One of them developed an ascites tumor. As mentioned above, original YB-9 tumor had diploid cells at 97.2%, but they decreased to 91.8% at the next transplant generation by usual transfer technique. The count of 250 metaphase cells in the ascites tumor developed by transfer of the hypertrophic lymphnode showed that the frequencies of diploid and tetraploid cells were at 93.2 and 6.8%, respectively. The frequency of diploid cells in this ascites tumor is a little higher than in the control batch. This ascites tumor was injected to intraperitoneal cavities of rats by usual transfer technique, and then the diploid cells decreased to 90.5%, but in the following transplant generation the number of diploid cells increased to 94.6% (Table 7 and Fig. 6). By

Table 7. Frequency of ploid cells in an ascites tumor developed by inoculation of hypertrophic lumbarnode

Transfer gener.	Ploidy				No. of cells observed	Strain of rat
	±2n	±3n	±4n	±8n		
Ascites tumor developed by inocul. of lymphnode	93.2	0.4	6.4	0	250	Wistar
1st transfer gener. of the above ascites tumor	90.5	0	9.5	0	42	Wistar
2nd transfer generation	94.6	2.2	3.2	0	317	Wistar

the above experiment it was confirmed that an increase of tetraploid tumor cells did not occur in the case of intraperitoneal transplantation of the hypertrophic lymphnode. It is quite possible that the lymphotrophy in tetraploid tumor cells was not stronger than that in diploid cells.

Discussion

Tumor cells have a remarkable ability to infiltrate into various organs of a tumor bearing host through blood and lymphatic system. Infiltrated cells can multiply in these organs. According to the present author's study (Yosida 1959), the affinity of tumor cells to organs varies by the difference of karyotype of tumor cells. Yosida

and Tsuji (1960) also observed that the affinity of the tetraploid tumor cells to lung tissue was much stronger than that of the diploid ones. Based on the examination of DNA content in primary and metastatic human tumor nuclei Rabotti (1959) concluded that the amount of DNA in the primary tumor nuclei showed the same value (2 DNA) as in the control lymphocytes, while in the metastatic lesions it was 4 DNA and 8 DNA. Similar observation was reported by Chu and Malmgren (1961) in primary and metastatic mouse tumors. According to them all the metastatic tumors tended to have a higher ploidy than the primary tumors from which they were derived. It is noticeable, that although the polyploid Yoshida sarcoma cells show a predominat preference to invade lung, spleen and kidney, but not thymus and lymphatic glands. Recently, a cytological study on original and metastatic human tumors was carried out by Maeda and Tabata (1960). They concluded that the metastatic tumors in lymphatic glands were characterized by predominance of neardiploid cells, notwithstanding the remarkable polyploid stemline characteristics of the original tumors. It seems highly possible that affinity to organs of tumor cells depends on their chromosomal constitution. It is possible that polyploid cells can penetrate easily to some organs through the blood system, and diploid cells have a preference to infiltrate through the lymphatic system.

Summary

In order to investigate the affinity of polyploid cells to various organs of rats bearing tumors, a common strain of Yoshida sarcoma in which polyploid cells were contained at a low percentage was used. Polyploid tumor cells showed much stronger affinity to spleen, kidney and lung, while the diploid ones showed stronger affinity to thymus and lymphatic glands. It was suggested that the polyploid cells can penetrate easier to organs through the blood system, but diploid cells infiltrate predominatly to lymphatic glands through the lymphatic system.

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[GANN, 52, 321~326; December, 1961]

CORRELATIVE HISTOCHEMICAL AND AUTORADIOGRAPHICAL STUDIES ON THE MOUSE SKIN AFTER THE PAINTING WITH TRITIUM LABELED 4-NITROQUINOLINE N-OXIDE

(With Plates XXXa and XXXb)

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Since the discovery of the potent carcinogenicity of 4-nitroquinoline N-oxide (Nakahara, Fukuoka and Sugimura[11]) the mode of interaction between it and cell component has become of great biological interest. According to Okabayashi[12], Endo[5] and Hayashi[7], the reaction between 4-nitroquinoline N-oxide and biological material is that of substitution with SH-group, which takes place rapidly within the physiological pH range and without enzymic intervention. It has been also known that under the similar condition 4-nitroquinoline N-oxide does not react with R-COOH, R-NH_2, ATP, RNA and DNA. In 1959[5], Endo reported that 4-nitroquinoline N-oxide produces characteristic intranuclear inclusion bodies in the tissue culture cells. The experimental data strongly suggest that 4-nitroquinoline N-oxide actually undergoes a reaction with some cellular components in the tissue culture.

The present study was attempted with the hope of finding out if 4-nitroquinoline N-oxide reacts with cellular components of the epidermal cells *in vivo* and how.

In this paper will be reported a correlative observation between histological-histochemical and autoradiographical findings on the mouse skin after painting with tritium-labeled 4-nitroquinoline N-oxide, and an analysis of intracellular localization of radioactivity the cellular components there.

In order to avoid trapping of active substance by the cornified layer of the mouse epidermis, which may have no direct effect on further proliferation of the epidermal elements, and in order to facilitate analysis of the activities of the differentiating cell layers, a single pretreatment with non-labeled 4-nitroquinoline N-oxide was given to the mouse skin before radioactive 4-nitroquinoline N-oxide was applied. The pretreatment is helpful to remove cornified surface layer and to induce multiplication of the epidermal cell layer.

Material and Methods

The sample of randomly tritiated 4-nitroquinoline N-oxide used was prepared by the New England Nuclear Corporation, Boston, U.S.A.. The specific activity of this substance was 0.042 millicuries per milligram.

Ten female mice of dd-strain were used, all weighing about 20 g and maintained on the standard synthetic diet in our laboratory.

They were divided into two groups. In *group 1*, each of 5 mice received a single dose of 0.02 ml of 0.5% solution of 4-nitroquinoline N-oxide in acetone on the interscapular dorsal skin by means of a small glass pipette. Seventy-two hours after the first treatment, 0.02 ml of 0.5% acetone solution of tritium-labeled 4-nitroquinoline N-oxide was dropped on the same area. Animals were sacrificed at 3, 6, 12, 24 and 48 hours after the second treatment.

In *group 2*, 5 mice were treated in the same way as in group 1, and the application of 0.02 ml of 0.5% acetone solution of tritium-labeled 4-nitroquinoline N-oxide was repeated once again 48 hours after the first application of the labeled compound. The animals were sacrificed at 24, 48 and 72 hours after the treatment.

The excised skin was placed on a sheet of filter paper and immediately fixed in 1% solution of trichloracetic acid in 80% ethanol for 24 hours. The paraffin sections were cut at 5 μ.

Histochemical procedures: For the histochemical detection of protein-bound sulfhydryl group Barrnett-Seligman's method[1] modified by Y. Hayashi[7] was used: 25 mg of DDD (2,2'-dihydroxy-6,6'-dinaphthyl disulfide) was dissolved in the solvent, prepared by mixing 25 ml of absolute ethanol with 25 ml of 0.1 M veronal buffer (pH 8.5). Other procedures were same as in the original method.

In order to facilitate exact comparison, normal and treated skin sections were mounted on the same slides and processed.

Autoradiographic procedures: Stripping method was applied for autoradiography. The paraffin sections were placed on glass slides coated with 0.5% gelatine*. After being sufficiently dried the sections were deparaffinized through xylene series. The slides thus prepared were covered with Kodak AR 10 autoradiographic stripping film by the method of Doniach and Pelc[4]. The slides were exposed for 45 days at 4°C. After development and fixation of the emulsion, Doniach and Pelc's staining method was adopted. Grain counts were obtained by running through a selected area under a high power light microscope at the magnification of 1,000 times.

For control, usual hematoxylin-eosin sections were prepared.

Results

Histological findings: *Group 1.* At 3 and 12 hours, the epidermis of the treated area showed slight acanthosis consisting of two or three layers of the cells. The surface was covered with cornified material. Occasionally ulcerous lesions were observed in the treated area. Dermal changes at this stage were vascular dilatation, edema and a diffuse round cell infiltration.

* 0.5% gelatine solution, containing 0.05% chrome alum.

At 48 hours, most parts of the epidermis in the treated regions were markedly thickened and the surfaces covered with hyper- and / or para-keratotic layers. Occasional superficial ulceration has occurred. Hair follicles and sebaceous glands were partly atrophied. Inflammatory changes in the dermis persisted.

Group 2. At 24 and 48 hours, the most conspicuous feature was remarkably thickened epidermis. Prominent acanthosis was often associated with slight papillary outgrowth on one hand and downward elongation of the rete pegs on the other. The surface was covered with hyper- and / or parakeratotic layers. Often regenerative processes of the epidermis were observed at the margin of the ulcer.

Histochemical findings: In the normal epidermis, the cytoplasm of the Malpighian layer cells stained moderately in purple. The granular layer cells showed an intensely purple coloration. However, the cornified layer reacted only slightly or showed no reaction.

Group 1. Contraly to the intact skin, the epidermis of the treated area at 3, 12 and 48 hours has become remarkably thickened, while the basal, spinous. granular and cornified layers were readily distinguished from each other in histochemical preparation using DDD. The basal cells were pale purple with the nucleoli staining rather prominently; the spinous cells appeared moderately in purple, showing more intensive reaction where they merged into the cornified layer; and the cornified layer showed an intense diffuse staining in blue, especially in the parakeratotic area. Diffuse and slight coloration occurred in the necrotic masses over the ulcerous lesions.

Group 2. In general, the histochemical findings in this group were quite comparable with those in group 1. The thin layer of the regenerative epidermal cells at the margin of the ulcers reacted with DDD just in the same way as the basal layer cells of the hyperplastic epidermis.

Autoradiographic findings: *Group 1.* At 3, 6 and 12 hours, the deposit of radioactive 4-nitroquinoline N-oxide was most prominent along the surface of the parakeratotic layer, and no radioactivity was found in the Malpighian layer. At 48 hours, the cornified layer has become thinner, along the surface of which the intense radioactivity was noticed. Where there were ulcerative processes, deposit of an intensely radioactive substance was noticed in the inflammatory exudates covering the ulcers.

Group 2. At 24 and 48 hours, the intense radioactivity still remained on the surface of the thin cornified layer and of the inflammatory exudates or masses.

At two particular sites, intracellular localization of radioactivity in the viable epidermal elements was clearly noticed in this group.

Moderate activity was seen in the epidermal cells surrounding the hair, whereas no incorporation of radioactive 4-nitroquinoline N-oxide has occured either in the

supra-bulbular regions, bulbs or keratinized parts of the hairs. An attempt to count grains in the epidermal cells of the above regions failed due to an extremely high incorporation.

An intense radioactivity was noticed in the regenerating epidermal cells at the ulcer margin. Grain counting covering an area of 30 regenerating cells in this part revealed that the grains exsisting almost exclusively in the areas corresponding to those of the nuclei. The average ratio of grain numbers over nuclear vs. cytoplasmic areas was 3.6 : 1, as illustrated in Chart 1.

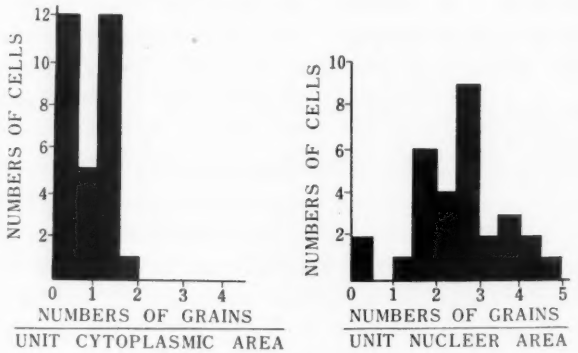


Chart 1. Comprison of number of grains per unit area between cytoplasm and nucleus.

Discussion

Hayashi (1960), using an improved Barrnett-Seligman' method, reported the histochemical localization of protein-bound SH-group in the mouse skin rendered hyperplastic by 20-methylcholanthrene painting. Most prominent reaction was shown in the parakeratotic layer and subjacent granular layer, while reaction in the spinous layer was less marked and quite weak in the basal layer. The fully keratinized layer was invariability negative in sharp contrast to the parakeratotic. His data obtained by a paralleled biochemical assay were quite comparable. The histochemical findings in the present experiment, in which hyperplasia was induced with 4-nitroquinoline N-oxide instead of 20-methylcholanthrene, are in full agreement with Hayashi's.

If 4-nitroquinoline N-oxide reacts with the SH-group *in vivo* without enzymic intervention, as has been proved by Okabayashi[12] and Endo[5], and if the labeled 4-nitroquinoline N-oxide homogenously reaches all sites in the epidermis where SH-groups are localized, it is expected that the autoradiographic pattern of the treated mouse skin should correspond to the histochemical pattern of SH-group.

In the present experiment, most of labeled 4-nitroquinoline N-oxide appeared to have been trapped in the superficial layer of the epidermis or in the exudates covering the basis of an ulceration, and not to have penetrated to the deeper layers

to be visualized by means of radioautography. The trapping has apparently occurred topographically in full accordance with histochemical localization of SH-group.

Only in group 2, however, considerable concentratian of radioactive material was observed in the limited area with spinous cells around the orifices of the hair-follicles and in the thin layer of regenerating epidermal cells at the margins of the ulcers. This suggests, under the experimental conditions, penetration of painted 4-nitroquinoline N-oxide to the more active epidermal components may be facilitated by the removal of the keratinized or parakeratinized layers overlying such components or even by inducing loss of continuity to the epidermis. The severe inflammatory reactions in the dermis after the initial paintings were proved not indicating penetration of the carcinogenic agent through the entire layers of the epidermis, much less to the dermis.

If ever it is true that 4-nitroquinoline N-oxide attains only a very limited penetration when painted on the skin, even after the procedure is repeated, then the role of the dermis in the epidermal carcinogenesis cannot be significant. On the other hand emphasis should be placed upon the significance of the regenerative process of the epidermis after ulceration. Further investigation may be necessary also concerning the implication of incorporated carcinogen into the perifolliclar epidermal elements.

Another interesting point emerging from the present findings is the almost exclusive localization of radioactivity in the nuclei of the epidermal cells in the paticular areas. The data are incomplete, however, as grain counting was possible only on the material at 24 hours.

There are following four possibilities: 1. Tritium labeled 4-nitroquinoline N-oxide was selectively incorporated into the nuclei. 2. The labeled agent was first transported into the cytoplasms, then transferred into the nuclei and remained there. 3. The carcinogen first penetrated into the nuclei and some of it being then transferred into the cytoplasms. 4. Radioactive 4-nitroquinoline N-oxide had been incorporated equally into the nucleus and the cytoplasm, but was quickely removed from the cytoplasm. There may or may not be a selective binding of substance with the nuclear material.

Time course observation should be necessary in order to clarify this point. But if 4-nitroquinoline N-oxide is to be selectively incorporated into the nuclei, it should present a very fascinating key to the solution of the mechanism involved in the carcinogenesis with 4-nitroquinoline N-oxide, because it has been reported with carcinogenic hydrocarbons that incorporation of such substances occurs invariably into the cytoplasm at the first stage.

Summary

1. Correlative histochemical and autoradiographical studies were done on the

mouse skin after the painting with tritium-labeled 4-nitroquinoline N-oxide.

2. A definite correlation was observed between the histochemical localization of SH-group and autoradiographical localization of the carcinogenic substance in the epidermis, although it was proved on the other hand that the substance does not usually penetrate deep enough and often becomes trapped in the superficial layers rich in SH-content.

3. The intense incorporation was observed in the regenerating cells, especially in the nuclei. The average ratio of the grain numbers over nuclear vs. cytoplasmic areas was 3.6 : 1.

We wish to express our hearty thanks to Dr. Waro Nakahara for his interest and encouragement throughout this study. Thanks are also due to Dr. Hiroto Naora and Dr. Yuzo Hayashi for helpful suggestions. We are indebted to Dr. Hideya Endo, now of The Kyusyu University Cancer Institute, whose generosity enabled us to use the tritium labeled 4-nitroquinoline N-oxide.

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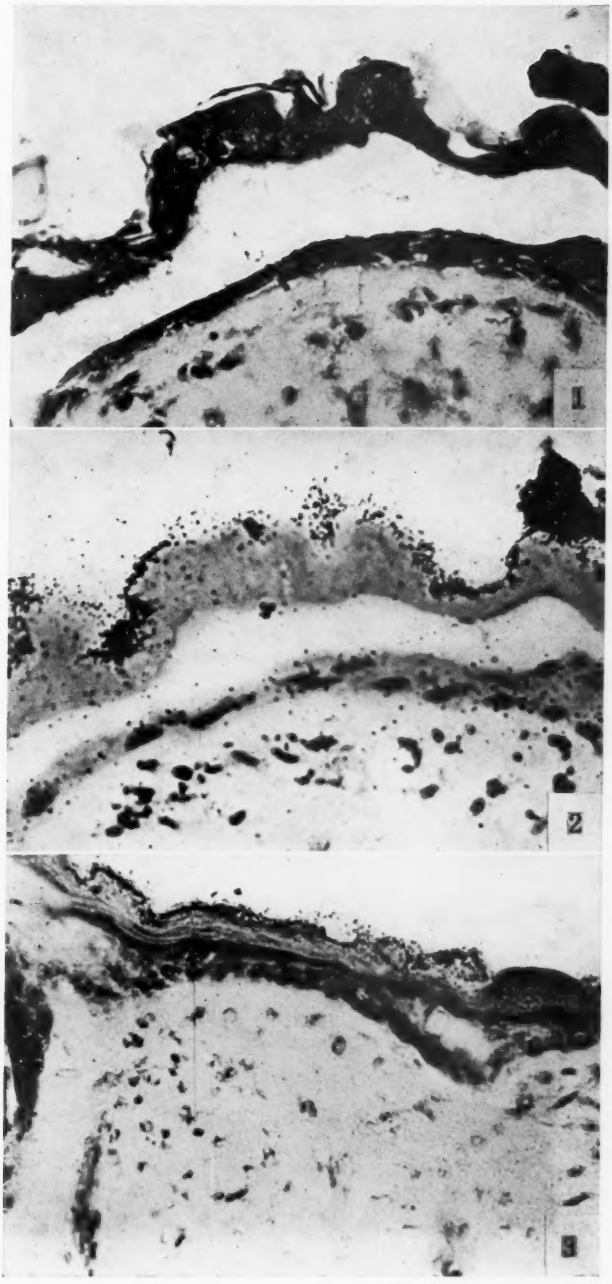
Explanation of PLATES XXXa and XXXb

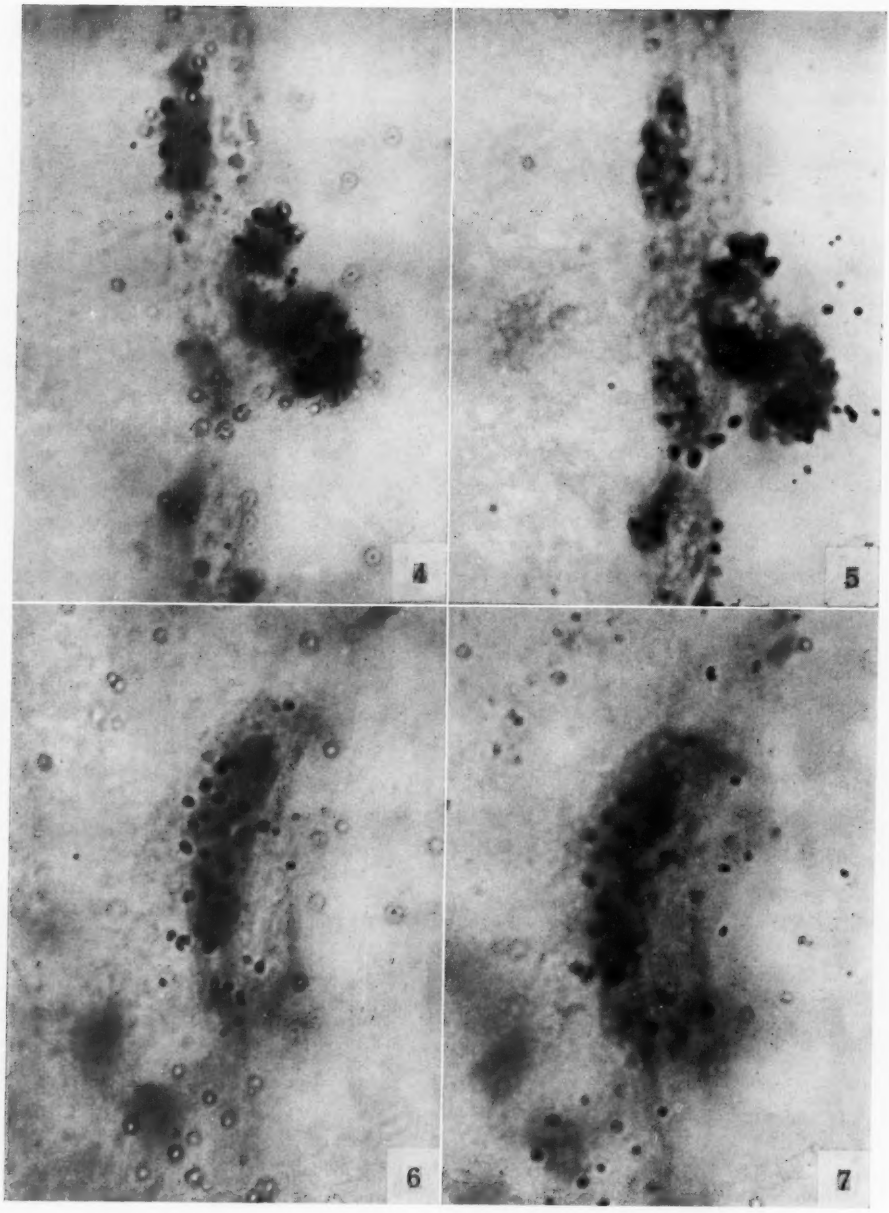
Fig. 1. Group 1, 48 hours after application of 0.5% of tritium labeled 4-nitroquinoline N-oxide in acetone. The parakeratotic layer of the acanthotic epidermis, loosened from the spinous layer, shows the most intense coloration along its surface. Histochemical demonstration of protein bound SH-group with DDD. 200×.

Fig. 2. Autoradiogram of a section of adjacent to Fig. 1. A prominent blackening is noticeable along the surface of the parakeratotic portions. 200×. Doniach & Pelc's staining.

Fig. 3. Group 2, 24 hours after application of hypertrophic epidermis. The grains are found exclusively along the surface of the parakeratotic layer. 200×. Doniach & Pelc's staining.

Figs. 4, 5, 6 and 7. (Group 2, 24 hours). Autoradiogram. The regenerating epidermal cells at the basis of an ulcer. In Fig. 4 & 6 the focuses are at the grain level, while in Fig. 5 & 7 at the section level. The grains are found exclusively over the nuclei. 1200×. Doniach & Pelc's staining.


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[GANN, 52, 327~335; December, 1961]

EFFECT OF TOXOHORMONE ON IRON-PROTOPORPHYRIN CHELATING ENZYME OF LIVER*

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In the course of the experiments to elucidate the mechanism of hepatic catalase depression in tumor-bearing animals and the animals injected with toxohormone, Ono *et al.* (1) observed the accumulation of free protoporphyrin in the blood and liver of these animals. These findings are considered to support the hypothesis that the heme synthesis in these animals is impaired at the step of iron insertion into protoporphyrin rings.

The impairment of heme synthesis may be due to various causes as recently reviewed by Nakahara (2). Above all, the important point to be studied is the enzymatic process of inserting iron into protoporphyrin in the liver of tumor-bearing animals and the animals injected with toxohormone.

Various studies on this enzyme catalyzing the insertion of iron into protoporphyrin from avian erythrocytes have been carried out by many investigators (3, 4, 5, 6, 7) but this enzyme in the liver has been scarcely investigated. Recently, however, Minakami *et al.* (9), Minakami (10), Nishida and Labbe (11) and Labbe (12) observed some properties of this enzyme. Afterwards Labbe and Hubbard (13) isolated this enzyme from rat liver mitochondria and reported many of its properties. Rimington and Tooth (14), also, reported some properties of this enzyme from rat liver mitochondria in their recent studies on the role of mitochondria in the *in vitro* formation of protoporphyrin and heme.

On the basis of these studies, which made evident the nature and the method of investigation of this enzyme in the liver, the present study was designed with view to elucidating the mechanism of impairement of heme biosynthesis at the step of iron insertion into protoporphyrin in the livers of tumor-bearing animals and of animals injected with toxohormone.

Materials and Methods

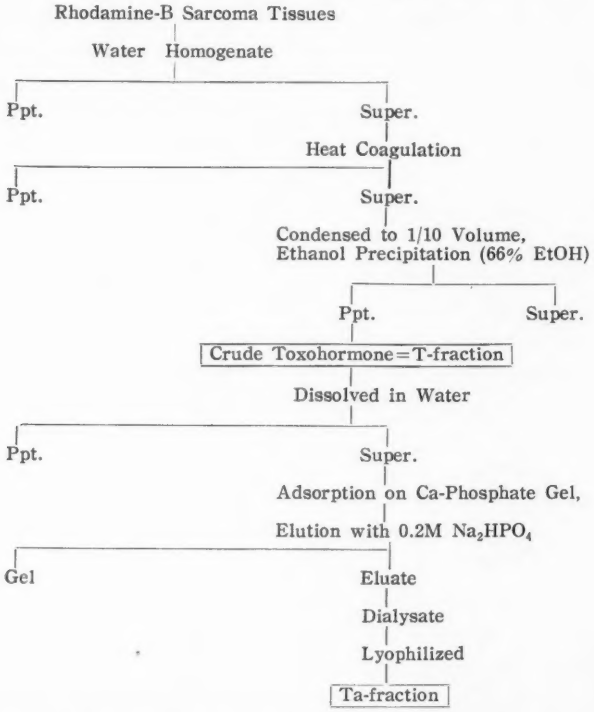
Toxohormone Preparation: In these experiments were used two kinds of toxohormone preparations which varied in the extracting procedures and activities, all

* This paper was presented at the 34th General Meeting of the Japanese Biochemical Society at Osaka, Nov. 4, 1961.

** Supported in part by grant from the Japan Science Council.

obtained from the rat rhodamine fibrosarcoma (Umeda). One of them, which is called T-fraction throughout this paper, was Nakahara and Fukuoka's alcohol precipitate and the second one called Ta-fraction was the fraction adsorbed on calcium triphosphate gel from T-fraction. The procedures of extracting these fractions are presented schematically in Table 1.

Table 1. Fractionation of Toxohormone.

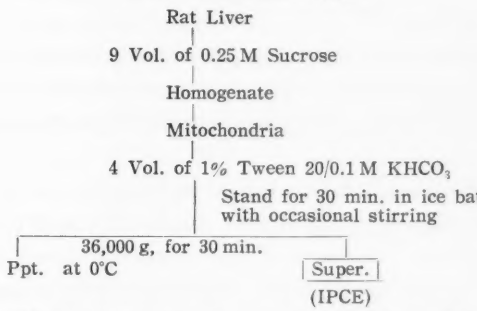


The activities of these preparations expressed as minimum dose per mouse to depress liver catalase activity *in vivo* were 75 mg and 20 mg for T- and Ta-fraction respectively, the more quantitative assay data being presented in the section of Results in this paper.

Measurement of IPCE (Iron-Protoporphyrin Chelating Enzyme) Activity : Preparation of the enzyme. The enzyme prepared from rat liver mitochondria according to Labbe and Hubbard (13) which is shown schematically in Table 2 was employed in the studies on the effect of toxohormone on it *in vitro*.

The enzyme from the animals injected with toxohormone or tumor-bearing animals, however, was prepared as follows; 10% of liver homogenate in M/15 phosphate buffer (pH 7.4) was prepared with Potter-Elvejem homogenizer and supernatant obtained by

Table 2. Preparation of Iron-Protoporphyrin Chelating Enzyme (IPCE). Labbe and Hubbard (13).



centrifugation at 4000 r.p.m. for 10 min. was employed as the enzyme solution.

Assay of IPCE Activity. The IPCE activity was determined essentially according to Labbe and Hubbard (13) by determining the disappearance of protoporphyrin used as a substrate.

Protoporphyrin was prepared by the method of Grinstein (15) from horse hemoglobin. It was further purified by dissolving in 0.15 M $KHCO_3$, centrifuged out insoluble material, and adjusting to pH 4-5 with glacial acetic acid. After its precipitation in cold, the protoporphyrin was centrifuged out, dissolved in 3 N HCl and insoluble material again centrifuged out. The protoporphyrin was precipitated with sodium acetate, washed with water and redissolved in 0.15 M $KHCO_3$ to a suitable concentration.

The detailed procedures of determining the enzyme activity were as follows: The enzyme, protoporphyrin, $FeSO_4$, Tris-buffer and with or without toxohormone in a total volume of 2.0 ml (The detailed quantities of these reagents were shown in the the section of Results in this paper) were incubated at 37°C for one hour. Following incubation 8.0 ml of 2 N HCl was added and the precipitated protein centrifuged out. The disappearance of protoporphyrin was measured at 407 mμ spectrophotometrically with Beckman DU spectrophotometer. A control mixture without enzyme was included in each assay and the amount of protoporphyrin consumed in the enzymic reaction was calculated from the decrease in absorption (ΔE_{407}).

Procedure of an assay using ^{59}Fe (radioactive iron) was performed as follows: Radioactive iron as $^{59}FeCl_3$ was purchased from the Oak Ridge National Laboratory in the United States. Usually 0.017 μc was added per sample containing 40 mμM protoporphyrin, 40 mμM $FeSO_4$, 80 μM ascorbate and 0.4 ml of enzyme from rat liver mitochondria (1.0 mg tyrosine equivalent) prepared as mentioned above in a total volume of 3.0 ml. Incubation was carried out at 37°C for one hour after the preincubation at the same temperature with or without Ta and the enzyme. Following incubation the labeled hemin was isolated through the use of a carrier according to

Nishida and Labbe (11). Radioactivity was measured with Kaken Well Type Scintilation Counter and the results expressed as counts / min. / mg hemin / mg tyrosine equivalent enzyme.

Measurement of Catalase Activity: The assay of liver catalase depressing activity of toxohormone was carried out using mice by the method employed routinely in this laboratory (16). That of the tumor-bearing animals was conducted also according to the same procedure (16).

Determination of Protein of IPCE: The amount of protein of IPCE from rat liver mitochondria was determined by the method of Lowry *et al.* (17) and expressed as tyrosine equivalent.

Tumor-Bearing Animals: Rats bearing Rhodamine-B fibrosarcoma, rats bearing ascites hepatoma 49 (AH 49) and mice bearing NF-fibrosarcoma were employed in this study. Time after transplantation of tumors, tumor weight and other detailed quantitative data are presented in the section of the results.

Results

Effect of Toxohormone (Ta) on Rat Liver IPCE *in vitro*: At the onset, the effect of Ta at various concentrations on the IPCE extracted from rat liver mitochondria was ascertained. As Labbe and Hubbard (13) reported previously that the enzyme was activated by ascorbate or other reducing reagents, the effect of Ta on this enzyme was examined both in the presence and absence of ascorbate. The enzyme and Ta were preincubated at 37°C for one hour in the presence of Tris buffer (pH 7.6), then protoporphyrin, $FeSO_4$ and ascorbate (in one case) were added and the

Table 3. Effect of Toxohormone (Ta) at various concentrations on rat liver IPCE *in vitro*.

Conditions: 40 mμM PROTO*., 40 mμM $FeSO_4$, 200 μM Tris-Buffer (pH 7.6), 0.5 ml of enzyme (1.25 mg tyrosine equivalent), Total Volume 3.0 ml. Enzyme+Ta+Buffer at 37°C for 1 hour. Then PROTO., $FeSO_4$ added.

Amount of Ta (mg)	IPCE Activity	
	Without Ascorbate PROTO. consumed mμM	With Ascorbate PROTO. consumed mμM
0	4.1	10.4
1	4.0	10.0
5	4.7	10.3
10	4.0	9.8
20	—	10.5
40	—	11.0

* PROTO.=Protoporphyrin.

Table 4. Effect of Ta on rat liver IPCE with ^{59}Fe.

Conditions: 40 mμM PROTO., 40 mμM $FeSO_4$, 0.017 μC (Total Counts, 55528 cpm) $^{59}FeCl_3$, 80 μM Ascorbate, 0.4ml of enzyme (1.0 mg tyrosine equivalent), Total Volume 3.0ml. Incubation at 37°C for 1 hour, after the preincubation at 37°C with Ta and enzyme. Hemin was crystalized after Nishida et al. (11). Radioactivity was measured with Kaken Well Type Scintilation Counter.

Amount of Ta (mg)	IPCE Activity cpm/mg hemin/mg tyrosine
0	138
1	140
5	137
10	138
20	142

enzyme activities were determined after incubation at 37°C for one hour.

As may be seen from Table 3, the inhibitory effect of Ta on IPCE was hardly observed both in the presence and absence of ascorbate, even when a large amount of Ta was added. In order to further ascertain the effect of Ta on IPCE, the IPCE activity was examined with the use of radioactive iron (^{59}Fe). The results are presented in Table 4, from which it is evident that the enzyme activities are scarcely affected by Ta even with this technique. Moreover, the enzyme activities were not altered by Ta even under different pH as may be seen from Table 5.

Table 5. Effect of Toxohormone (Ta) on rat liver IPCE at various pH.

Conditions: 40m μM PROTO., 40 mμM $FeSO_4$, 200 μM Tris-Buffer (pH 6.5–8.5), 0.5ml of enzyme (1.25 mg tyrosine equivalent), Total Volume 2.0 ml.

Incubation at 37°C for 1 hour, after the preincubation at 37°C with 5 mg Ta and enzyme at various pH.

pH	IPCE Activity (PROTO. utilized mμM)	
	Ta, 5 mg	Control
6.5	3.7	3.7
7.6	4.0	4.1
8.5	3.8	3.7

Effects of Toxohormone (T) on Mouse Liver IPCE and Catalase *in vivo*.

In this experiment, 80 mg of T-fraction was injected intraperitoneally into mouse and the liver catalase and IPCE activities were measured at various times after injection. Catalase activities were assayed according to the method of von Euler and Josephson (16) with 0.1 ml of 1% liver homogenate, whereas IPCE activities were assayed by the usual method of determining spectrophotometrically the disappearance of protoporphyrin with 0.5 ml of 10% liver homogenate. Even two hours after injection of toxohormone, catalase activity was decreased 30% that of control, whereas IPCE activity was entirely not affected. From 2 hours on, catalase activity further

Table 6. Effects of Toxohormone on mouse liver IPCE and Catalase *in vivo*.

Conditions: 40 mμM PROTO., 40mμM $FeSO_4$, 200 μm Tris-Buffer (pH 7.6), 0.5 ml of liver homogenate (1/15M Phosphate Buffer, pH 7.4), H_2O to 2.0ml.

Incubation at 37°C for 1 hour; Injection of Toxohormone, 80 mg/head mouse weighing 20±3 g.

Time after injection (Hours)	Number of mice	Catalase Activity*		IPCE Activity PROTO. consumed mμM
		K/min.	% Decrease	
2	5	0.167±0.027	30.4	9.9±0.7
4	5	0.159±0.019	33.6	9.7±0.1
6	5	0.143±0.023	40.4	8.9±1.0
13	5	0.128±0.034	48.1	8.9±0.6
24	5	0.120±0.020	50.0	8.8±0.7
Control	5	0.240±0.031	—	9.9±0.7

* Euler-Josephson method (16), 0.1 ml of 1% liver homogenate used as the enzyme sol.

decreased gradually and the activity at 24 hours after injection was 50% lower than that of control. On the other hand, IPCE activity was scarcely inhibited although from 6 hours on after injection of toxohormone only insignificant depression of the enzyme activity was observed. Thus it becomes evident that the inhibition of IPCE by toxohormone does not take place even *in vivo* (Table 6).

IPCE and Catalase Activities of Tumor-Bearing Animals.

With a view to further elucidating the mechanism of disturbances of catalase biosynthesis by the toxohormone released from tumor tissue, the activities of liver IPCE and catalase were examined in the various tumor-bearing animals listed in Table 7. Rats bearing Rhodamine fibrosarcoma, which is the source of toxohormone

Table 7. IPCE and Catalase Activities of Tumor-Bearing Animals.

Conditions: 40 mμM PROTO., 40 mμM $FeSO_4$, 200 μM Tris-Buffer (pH 7.6), 0.5ml of 10% liver homogenate (M/15 Phosphate Buffer (pH 7.4)), H_2O to 2.0 ml.
Catalase Activities: Euler-Josephson method (16), Enzyme Amount; rat, 0.1 ml of 2% liver homogenate; mouse, 0.1 ml of 1% liver homogenate.

Animals and Tumors	Number of Animals	Time after Transplantation (days)	Body Weight (g)	Tumor Weight (g)	Catalase Activity (K/Min.)	IPCE Activity (mμM PROTO.)
Rats with Rhodamine-B Sarcoma	5	25	249±11	56.7±14.5	0.123±0.083 (57.6)*	11.2±0.7
Rats with AH 49	5	9	260±20	—	0.194±0.017 (32.9)	10.4±0.5
Normal Rats	5	—	237±21	—	0.289±0.033 (—)	11.3±0.3
Mice with NF-Sarcoma	5	16	20.0±1.2	0.9±0.5	0.094±0.002 (42.0)	13.4±0.1
Normal Mice	5	—	19.4±1.1	—	0.163±0.040 (—)	13.5±0.8

* % decrease

employed in our experiments, showed marked depression of liver catalase activity, while the liver IPCE activity was scarcely altered. These results, therefore, agree with those obtained in the preceding experiments employing toxohormone isolated from the similar Rhodamine fibrosarcoma of rat. The depression of IPCE activity of rat bearing AH 49 and mouse bearing NF-fibrosarcoma were not observed as in the case of rat bearing Rhodamine sarcoma although marked depression of liver catalase was observed in both animals.

Discussion

From the experiments mentioned above, it is evident that the activity of liver IPCE is not inhibited by toxohormone either *in vitro* or *in vivo*. Furthermore, the enzyme activity was not inhibited in the tumor-bearing animals. Therefore accumulation of free protoporphyrin in the livers of tumor-bearing animals and the animals injected

with toxohormone may not be due to the direct inhibition of IPCE. It seems to be caused by other mechanisms.

The outstanding systemic changes so far established in tumor-bearing animals and in the animals injected with toxohormone are the disturbances of iron metabolism. The depression of liver catalase synthesis, with corresponding increase of protoporphyrin as mentioned above (1), and the decrease of liver ferritin (18) and of plasma iron (19, 20, 21, 22, 23) seem to be closely interrelated phenomena, and as such they speak strongly for the implication of toxohormone in the disturbed iron metabolism in tumor-bearing animals. The marked depression of liver ferritin after an injection of toxohormone (18) is worthy of attention, because iron released from ferritin is considered to be incorporated into protoporphyrin catalyzed by IPCE. In other words, if the iron supply from ferritin is depressed the heme formation may be directly injured even if all the other factors in the heme forming system are normal. This hypothesis was further strengthened by the iron administration experiments, in which a large dose of iron in the diet completely prevented the free protoporphyrin accumulation in the liver of tumor-bearing animals and the iron administration combined with cobalt corrected completely the anemia of tumor-bearing animals (24, 25).

According to Nishida and Labbe (11) the IPCE activity in the rat liver is stimulated by both forms of reduced and oxidized di- and triphosphopyridine nucleotides and reduced glutathione. Furthermore, Labbe and Hubbard (13) reported that the enzyme in the rat liver mitochondria exhibits numerous properties of an enzyme requiring a sulfhydryl group for activity and its activity is stimulated by various reducing reagents and DPN. In this connection works done by several investigators on the metabolism of DPN, TPN and ascorbate in the liver of the tumor-bearing animals and the animals injected with toxohormone are worthy of attention.

Waravdekar *et al.* (26) first reported that the DPN-synthesizing activity of the liver was lower in tumor-bearing animals than in normal controls, and suggested the existence of humoral factor responsible for this change. Ono and Tomaru (27) demonstrated that the increase of DPN in the liver, induced by nicotinamide administration, was markedly inhibited 24 hours after an injection of toxohormone in doses sufficient to depress the liver catalase activity. However, the tumor and toxohormone effect on the synthesis of liver DPN is limited to the inhibition of the synthesis *in vivo* and not observed *in vitro*. According to Glock and Mclean (28), although liver from tumor-bearing rats has a normal DPN content, the total TPN content is less than one-half that of normal liver. These results suggest, at least, the depression of TPN stimulating IPCE in the livers of tumor-bearing animals and the animals injected with toxohormone, although effect of toxohormone on TPN metabolism in liver have not been studied. On the other hand, Hoshizima (29) reported that toxohormone

prepared by the method of Ono *et al.* (acetic acid -methanol extraction) (30) induced a parallel depression of total ascorbic acid and catalase of mouse liver. Toxohormone prepared by the original method of Nakahara and Fukuoka (water extraction and then alcohol precipitation) showed no effect on the level of liver ascorbic acid, but it depressed catalase activity and ferritin iron, although the extent of their depression was not in parallel. The discrepancy of the depressing action on the liver ascorbic acid content between these two toxohormone preparations may probably be due to the degree of purity.

The depression of these cofactors responsible for the IPCE activity in the tumor-bearing animals or the animals injected with toxohormone, however, may not be so marked, because the enzyme activities of tumor-bearing animals or the animals injected with toxohormone were hardly depressed compared with those of control animals. The disturbances of iron metabolism, above all the depression of the source of iron to synthesize heme may be the fundamental cause of injury of heme biosynthesis at the step of iron insertion into protoporphyrin. However, many problems on this mechanism still remain to be solved.

Summary

The effect of toxohormone prepared from Rhodamine-B fibrosarcoma on liver IPCE was studied both *in vitro* and *in vivo*. The activity of this enzyme was not depressed in both cases. Furthermore it was not affected in the tumor-bearing animals. Injury of heme biosynthesis at the step of iron insertion into protoporphyrin in the tumor-bearing animals or in the animals injected with toxohormone is not due to the inhibition of IPCE but to other factor or factors.

Our hearty thanks are due to Dr. Waro Nakahara for his continued encouragement and giudance. Thanks are also due to Mr. Kiyoshi Noguchi for his technical assistance.

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[GANN, 52, 337~341: December, 1961]

ON HISTOCHEMICAL DIFFERENCES AMONG NORMAL, PRECANCEROUS AND CANCEROUS TISSUES: A STUDY WITH MITOCHONDRIA AND RIBONUCLEIC ACID STAINS

(With Plates XXXI—XXXVII)

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In the normal organism, the individual epithelial tissue groups have specific functions, which differ from one another in the individual organs or, with more pertinent expression, in the individual cell groups. For example, the parietal cells of the fundic gland of the stomach secrete hydrochloric acid, the chief cells secrete pepsin and the mucous cells mucus. A representative of such secretory organs may be the pancreas. Here, by massive protein synthesis, abundant potent digestive enzymes are produced and secreted. Secretion is of course one of the innumerable categories related to the various functions of the epithelial cells, among which protection, absorption, synthesis, detoxication, storage, secretion and excretion are the most important ones. For these functions a specific energy source is necessary. This energy is, apart from the energy necessary for the maintenance of the life of the cells themselves, supplied by decomposition of the glucose (glycolysis) transported by the blood. In tumor tissues these specific functions are reduced or stopped and, instead, marked growth or/and infiltration predominate, i.e., an acquired ability to migrate into neighboring tissues. It is certain that in malignant tissues, in parallel with the cessation of normal functions, the energy, which normally is used for specific purposes, is diverted to other functions.

At the 14 th General Meeting of the Japanese Medical Association in 1954 one of the authors (M.K.)[12] gave a summary of various aspects of precancerous lesions with special referrence to those of the stomach, rectum and breast. The precancerous lesions of the stomach can be divided into polypi, deep ulcers, shallow but large ulcers, atrophic gastritis with a protracted healing time and some kinds of congenital malformations. In the rectum they include polypi, polyposis and some chronic ulcerative lesions, and in the breast some of the features seen in chronic cystic mastopathy, such as intraductal papillomata. In general, adenomata and papillomata may be regarded as the most important clinical entities between normal tissuse and cancers. On the other hand, ulcerative lesions with long clinical courses often have

a tendency to develop malignant transformations. It is self-evident that aberrant tissues are more inclined to cancerous transformation than normal tissues.

Most of these pathological lesions have special clinical signs so that they can be detected by precise clinical investigations. Moreover, surgeons with ample clinical experience can distinguish the existence or co-existence of precancerous lesions in surgical specimens by macroscopical observation. This is especially important for this kind of investigation, since for certain specific staining, fixation by appropriate solutions is required. As soon as materials were obtained, those parts which were suspected to be precancerous were isolated. Half this tissue was fixed in appropriate solutions for specific staining and the other half was fixed in formalin for non-specific staining. Normal tissue and carcinomatous tissue were treated similarly.

The organs and tissues examined in the present investigations included*: skin, skin around the anus and penis, the mucous membrane of the buccal cavity (Watanabe[22]) stomach and duodenum (Sugita[17] and Kawahara[10]), colon and rectum (Tomita[19]), pancreas (Nakamura[15]), liver (Aizawa[1] and Kaneki[9]), urinary bladder (Watanabe[22],) mammary gland (Taniguchi[18], Yasui[23], Ooue[16], Murakami[14] and Morishita), the lung (Wakasugi[20]) and the thyroid gland (Magoshi[13]). In the tongue, stomach, duodenum, colon, rectum, and urinary bladder adenomata or papillomata were easily distinguishable from both normal tissue and from cancerous foci. Among various features found in chronic cystic mastopathy, intraductal papillomata were chosen as a precancerous change of the breast. About 80 lobectomy specimens of the lung were examined in detail with special referrence to tuberculous cavities. Several papillomatous hyperplasias were found in bronchioli near the cavities and these were examined in detail as precancer of the lung. In cysts of nodulary goiter, papillary growth is not infrequently observed. These were chosen as a precancerous lesion of the thyroid.

The recent progress in biological chemistry and histochemistry has resulted in some degree of clarification of the biological significance of various specific substances which can be distinguished with appropriate stain. For example, stains have been developed for study of sugar metabolism, fat metabolism, protein metabolism and for components of the TCA cycle. In the present study dozens of different stains were utilized to study pathological metabolism in tumor tissue. Here special attention was paid to the problem of whether there were any differences in common between normal tissue, precancerous hyperplasia and cancer which could be recognizable, irrespective of differences in the tissues of the various organs. Two stains were found which gave similar results in different organs. These are the stains for mitochondria and for ribonucleic acid. We would like to report about these two here.

Mitochondria are characteristic structures in the cytoplasm which were first

* The names of co-workers are shown in parentheses after each organ.

demonstrated in the last century by Flemming[6] and by Altmann[2]. They have recently acquired special biochemical significance and have been investigated in detail. Various important enzymes were demonstrated in them, such as phosphorylase (Claude[4]), oxidase (Hogeboom[7]), fatty acid oxidase (Kennedy[11]) and so on (Green[5], Hogeboom[8]). The substance which stains red with Unna-Pappenheim's pyronine-methylgreen stain has been identified as ribonucleic acid, since it is selectively decomposed by ribonuclease. Similarly, the substance which stains green with this stain has been shown by treatment with deoxyribonuclease to be deoxyribonucleic acid (Brachet[3]).

In normal cells mitochondria are mainly fine and granular or filamentous and are dispersedly distributed in the cytoplasm (Figs. 1, 7, 19, 25, 31, 37). They are almost constant in number. In the surface epithelia showing intestinal metaplasia not infrequently observed in pathological gastric mucosa, the form of mitochondria is scarcely changed (Fig. 9). In precancerous lesions, they are large and granular or club-like and markedly increased in number (Figs. 2, 8, 20, 26, 32, 38), also in proliferating stage of metaplasia of gastric mucosa (Fig. 13). This feature is also observable in the initial stages of cancer, especially in adenocarcinoma with high cylindrical cells (Figs. 14, 21, 33, 39) or in squamous cell carcinoma with marked pearl formation (Fig. 3). However, such increase in mitochondria can be no longer found in highly anaplastic carcinoma with an advanced tendency to infiltration. In such cancers, the mitochondria are fewer in number and smaller in size (Figs. 15, 27). In extreme cases only nuclear substances are found and the mitochondria are scarcely recognizable. As is well known in glandular structures the form and number of mitochandria differ greatly in relation to secretion. Therefore, it is only possible to compare them in different cells at a similar stage of secretion. In our investigation which was done exclusively with material obtained at operation, there had always been a previous 12 hours period of starvation before the operation, so that corresponding figures could be obtained.

The second common difference was found when the ribonucleic acid was studied. Common to all organs examined, in normal epithelia, the ribonucleic acid stained diffusely and weakly in the cytoplasma (Figs. 4, 10, 22, 28, 34, 40), the feature of ribonucleic acid in the intestinal metaplasia of stomach is a little deep stained and arranged apical to the nucleus (Fig. 12). In the precancerous conditions, it always stained deeper and seemed to have increased in amount (Figs. 5, 11, 23, 29, 35, 41), also in proliferating stage of intestinal metaplasia of stomach (Fig. 16). In cancerous cells it stained most deeply and was especially concentrated around the nucleus (Figs. 6, 17, 18, 24, 30, 36, 42).

How can these two differences be explained? It is certain that the final stage of the precancerous condition coincides with the initial stage of cancer or with cancer

in situ. The most significant difference which distinguishes precancer from real cancer may be heterotopic growth, that is to say infiltration and metastasis. In fact, in certain types of the so-called carcinoma simplex, rapid growth is less conspicuous and infiltration preponderates. As we pointed out at the beginning of this paper, infiltration is simply the movement of isolated cancer cells into neighbouring tissues. Therefore, if we classify the stages of transformation between normal epithelia and fully developed malignancy, then the first stage may be characterized by the abolition of normal function and the transmission of energy thus left over into growth. Precancer, as well as the initial stages of cancer, may belong to this. In the second stage the cancerous cells acquire an ability to migrate, so that their energy consumption undergoes a marked transformation with a subsequent change in the metabolism of the cells.

The difference in the mitochondria and ribonucleic acid in cancer and precancer may be explained to some extent along these lines. The synthesis of ribonucleic acid increases steadily with the development of anaplasia, while on the contrary, the mitochondria, where most of the intracellular respiration occurs, decrease in the fully developed malignant stage after a temporary increase in the precancerous stage. These anatomical deta may be in accordance with the biochemical concept that the energy in cancerous cells is not obtained from the terminal respiratory system but chiefly from anaerobic glycolysis (Warburg[21]).

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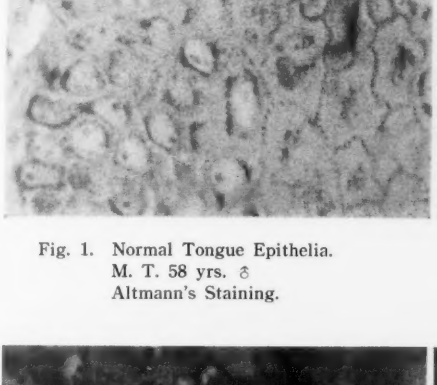


Fig. 1. Normal Tongue Epithelia.
M. T. 58 yrs. ♂
Altmann's Staining.

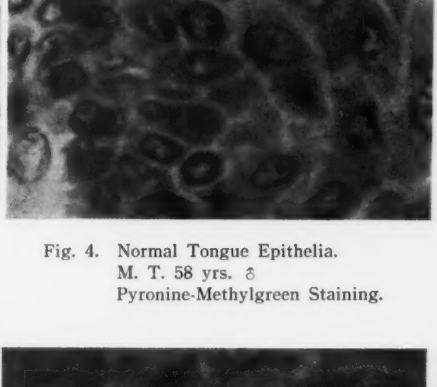


Fig. 4. Normal Tongue Epithelia.
M. T. 58 yrs. ♂
Pyronine-Methylgreen Staining.

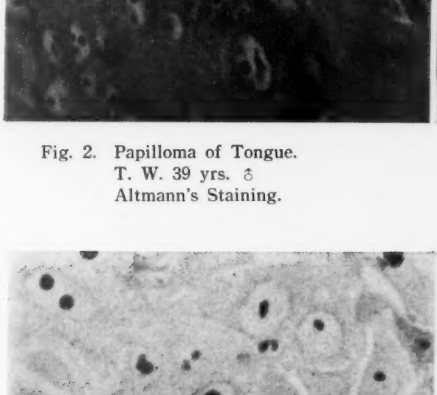


Fig. 2. Papilloma of Tongue.
T. W. 39 yrs. ♂
Altmann's Staining.

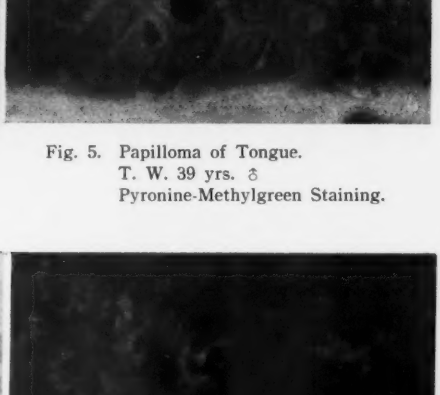


Fig. 5. Papilloma of Tongue.
T. W. 39 yrs. ♂
Pyronine-Methylgreen Staining.

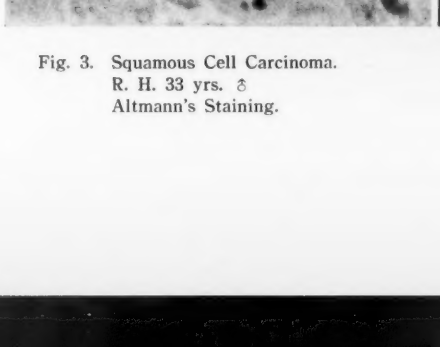


Fig. 3. Squamous Cell Carcinoma.
R. H. 33 yrs. ♂
Altmann's Staining.

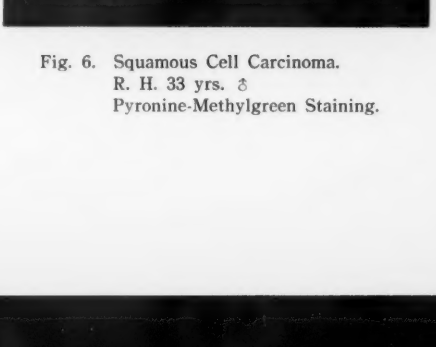


Fig. 6. Squamous Cell Carcinoma.
R. H. 33 yrs. ♂
Pyronine-Methylgreen Staining.

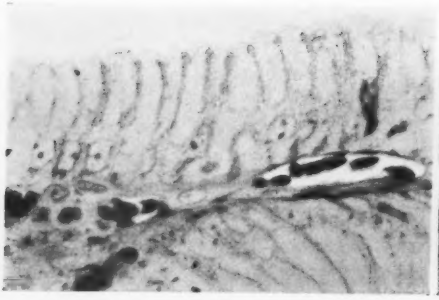


Fig. 7. Normal Surface Epithelia of Stomach.
S. T. 50 yrs. ♂
Altmann's Staining.



Fig. 10. Normal Surface Epithelia of Stomach.
M. N. 44 yrs. ♂
Pyronine-Methylgreen Staining.



Fig. 8. Hyperplasia of Surface Epithelia of Stomach.
K. F. 34 yrs. ♂
Altmann's Staining.



Fig. 11. Hyperplasia of Surface Epithelia of Stomach.
Y. T. 48 yrs. ♂
Pyronine-Methylgreen Staining.

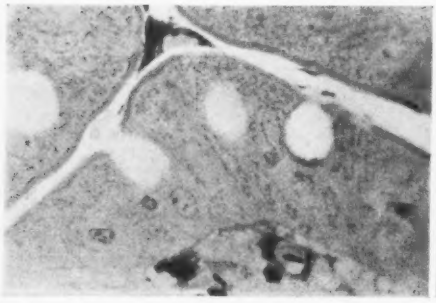


Fig. 9. Intestinal Metaplasia of Gastric Mucous Membrane.
S. T. 50 yrs. ♂
Altmann's Staining.

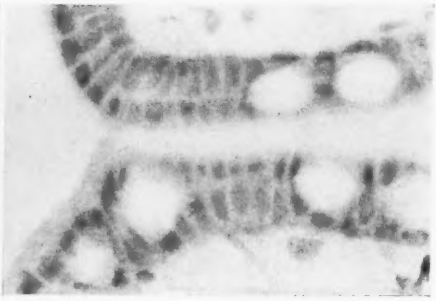


Fig. 12. Intestinal Metaplasia of Gastric Mucous Membrane.
K. M. 52 yrs. ♀
Pyronine-Methylgreen Staining.

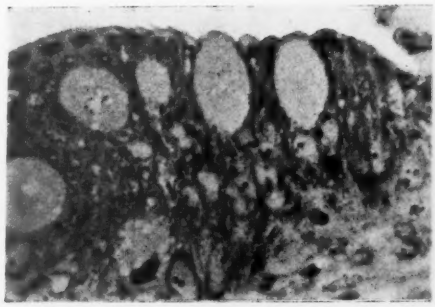


Fig. 13. Proliferating Intestinal Metaplasia of Gastric Mucous Membrane.
S. T. 50 yrs. ♂
Altmann's Staining.

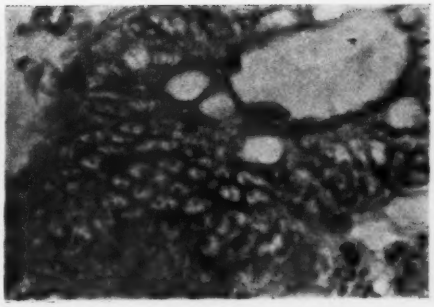


Fig. 16. Proliferating Intestinal Metaplasia of Gastric Mucous Membrane.
K. M. 52 yrs. ♀
Pyronine-Methylgreen Staining.

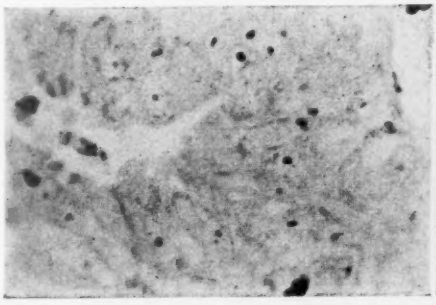


Fig. 14. Adenccarcinoma of Stomach.
K. N. 48 yrs. ♂
Altmann's Staining.

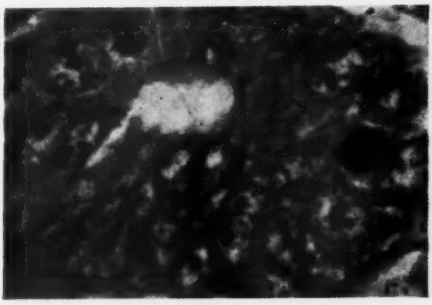


Fig. 17. Adenocarcinoma of Stomach.
K. N. 48 yrs. ♂
Pyronine-Methylgreen Staining.

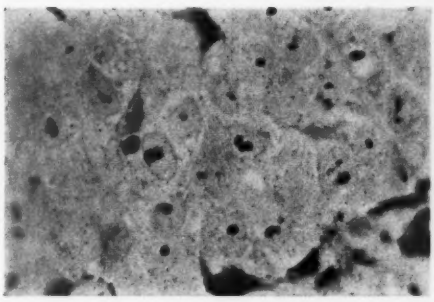


Fig. 15. Medullar Cancer of Stomach.
H. H. 59 yrs. ♀
Altmann's Staining.

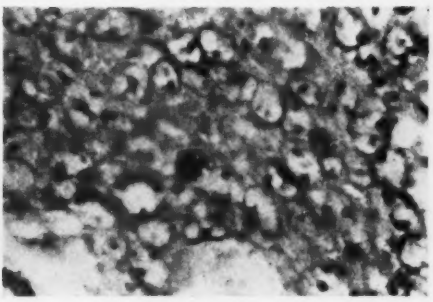


Fig. 18. Medullar Cancer of Stomach.
H. H. 59 yrs. ♀
Pyronine-Methylgreen Staining.



Fig. 19. Normal Epithelia of Rectum.
Y. O. 54 yrs. ♂
Altmann's Staining.



Fig. 22. Normal Epithelia of Rectum.
Y. O. 54 yrs. ♂
Pyronin-Methylgreen Staining.

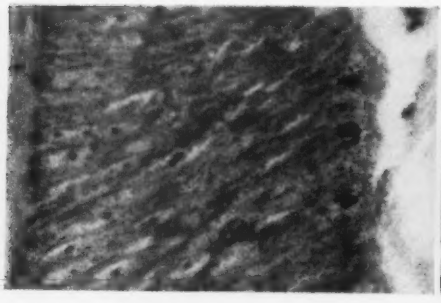


Fig. 20. Rectal Polyp.
K. O. 65 yrs. ♂
Altmann's Staining.

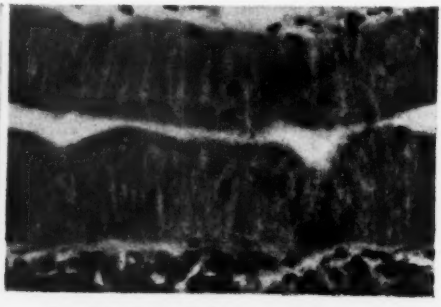


Fig. 23. Rectal Polyp.
C. M. 68 yrs. ♀
Pyronine-Methylgreen Staining.

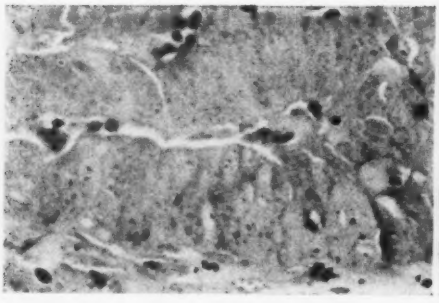


Fig. 21. Adenocarcinoma of Rectum.
T. M. 68 yrs. ♀
Altmann's Staining.

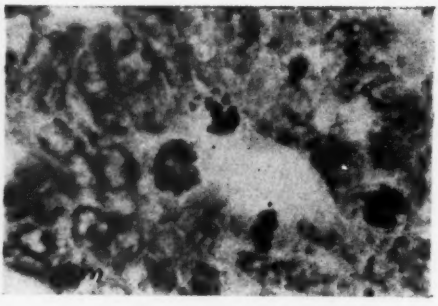


Fig. 24. Adenocarcinoma of Rectum.
R. N. 64 yrs. ♀
Pyronine-Methylgreen Staining.



Fig. 25. Normal Mammary Gland.
Y. I. 24 yrs. ♀
Altmann's Staining.

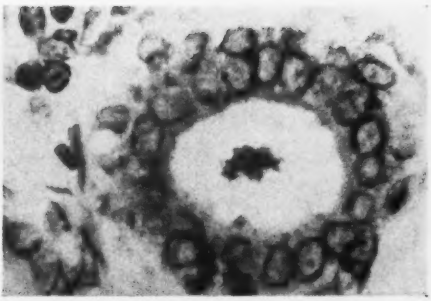


Fig. 28. Normal Mammary Gland.
Y. I. 24 yrs. ♀
Pyronine-Methylgreen Staining.



Fig. 26. Papilloma of Mammary Gland.
M. S. 42 yrs. ♀
Altmann's Staining.

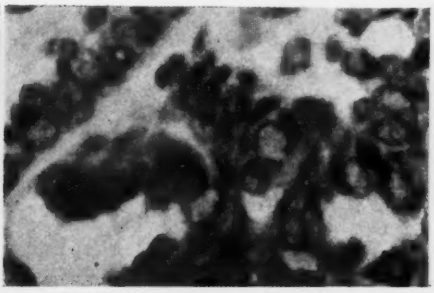


Fig. 29. Papilloma of Mammary Gland.
M. S. 42 yrs. ♀
Pyronine-Methylgreen Staining.

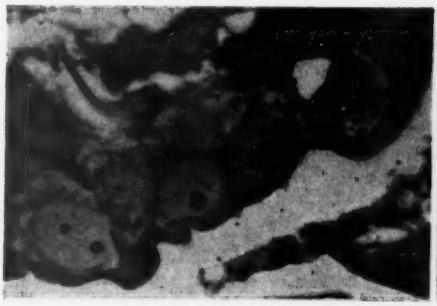


Fig. 27. Scirrhous Carcinoma of Mammary Gland.
M. I. 38 yrs. ♀
Altmann's Staining.

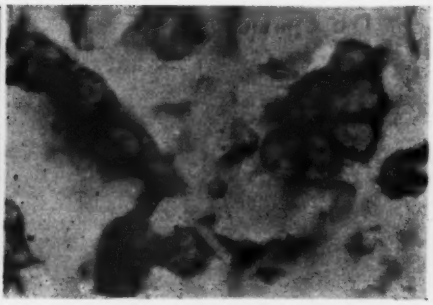


Fig. 30. Scirrhous Carcinoma of Mammary Gland.
M. I. 38 yrs. ♀
Pyronine-Methylgreen Staining.

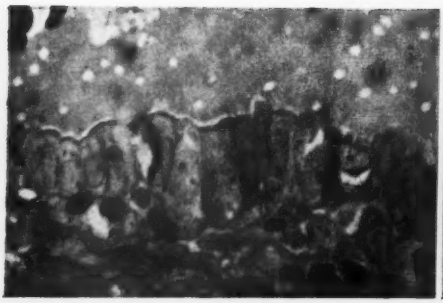


Fig. 31. Normal Bronchioli.
H. N. 31 yrs. ♂
Altmann's Staining.

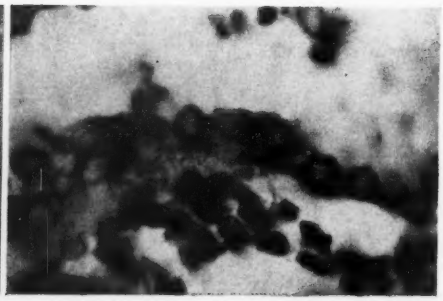


Fig. 34. Normal Bronchioli.
Y. U. 30 yrs. ♂
Pyroninine-Methylgreen Staining.

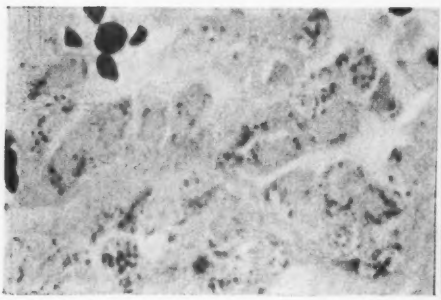


Fig. 32. Proliferating Regenerating Epithelia of Bronchioli.
S. T. 23 yrs. ♂
Altmann's Staining.



Fig. 35. Proliferating Regenerating Epithelia of Bronchioli.
Y. U. 30 yrs. ♂
Pyronine-Methylgreen Staining.

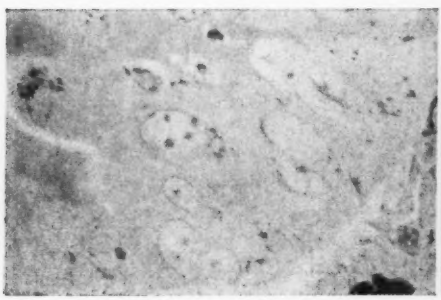


Fig. 33. Bronchogenic Carcinoma.
C. I. 48 yrs. ♀
Altmann's Staining.

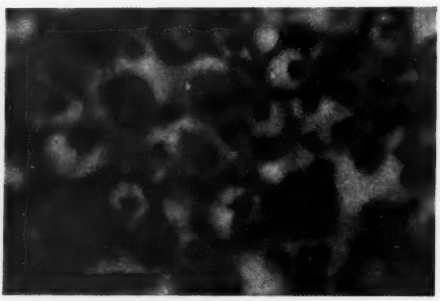


Fig. 36. Broncogenic Carcinoma.
K. H. 45 yrs. ♂
Pyronine-Methylgreen Staining.

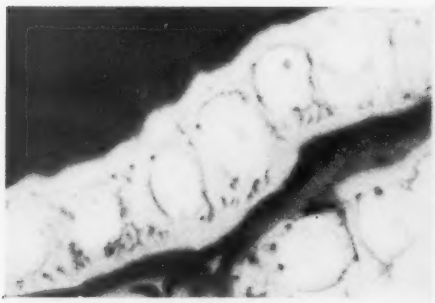


Fig. 37. Normal Follicular Epithela of Thyroid Gland.
A. H. 25 yrs. ♀
Altmann's Staining.

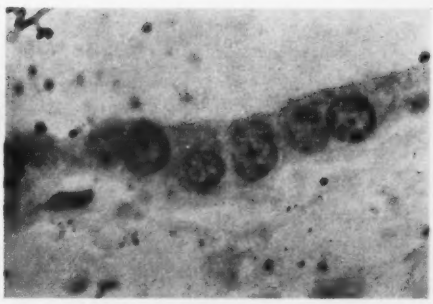


Fig. 40. Normal Follicular Epithelia of Thyroid Gland.
M. N. 22 yrs. ♀
Pyronine-Methylgreen Staining.

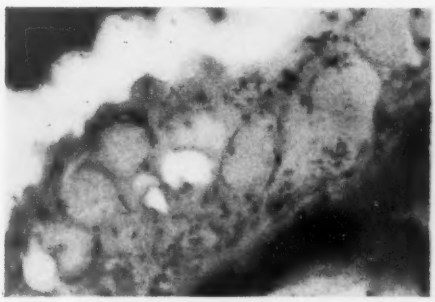


Fig. 38. Proliferating Follicular Epithelia of Thyroid Gland.
A. H. 25 yrs. ♀
Altmann's Staining.

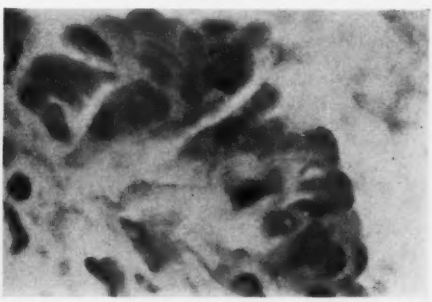


Fig. 41. Proliferating Follicular Epithelia of Thyroid Gland.
A. H. 25 yrs. ♀
Pyronine-Methylgreen Staining.

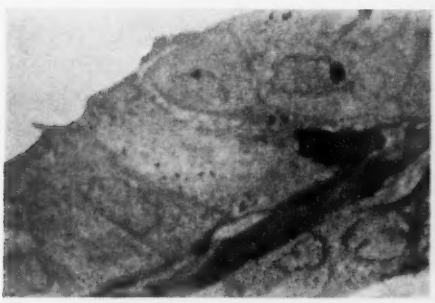


Fig. 39. Follicular Adenocarcinoma of Thyroid Gland.
Y. U. 59 yrs. ♀
Altmann's Staining.



Fig. 42. Anaplastic Adenocarcinoma of Thyroid Gland.
Y. U. 59 yrs. ♀
Pyronine-Methylgreen Staining.

[GANN, 52, 343~345; December, 1961]

THE EFFECT OF ONCOTREPHIN UPON INCORPORATION OF ^{14}C-OROTIC ACID INTO RNA OF NORMAL LIVER SLICES *IN VITRO*

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SEIZI YASUDA and MASAHIRO OZIRI
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Osaka University Medical School)

In the previous communications (Kuru *et al.*, 1~6) the existence of a mitosis promoting substance in certain rapidly growing malignant tumors was reported. This substance accelerates the mitosis of rat pinna epidermis *in vitro* and promotes the growth of strain L cells in tissue culture (Kuru *et al.*, 1~4). It is heat stable, non-dialysable and can be precipitated by 30–70% ethanol from saline extracts of homogenates of either human malignant tissues or rat ascites hepatoma. The further electrophoretic analysis of the precipitate at 30—70% ethanol has shown that the effective substance is in a fraction which migrates a little slower than human β-globulin at pH 8.6 (Kuru *et al.*, 5.). The activity of the effective substance is abolished by HCl hydrolysis and by pronase treatment, but not by trypsin treatment (Kôsaki *et al.*, 6.). Since on trypsin treatment the effective substance becomes partially dialysable, the minimal effective unit is thought to be a peptide. The effective substance was named oncotrephin.

In general accelerated cell division anticipates accelerated synthesis of nucleic acid and protein. Therefore, studies were made on the effective substance in the ethanol precipitate to see if it accelerated synthesis of nucleic acid *in vitro*.

Materials and Methods

A crude preparation of oncotrephin was made from a human hepatoma obtained by hepatectomy and from rat ascites hepatoma (AH 130) by the procedure described in the previous papers (Kôsaki *et al.*, 2.).

Experiments were performed by a modification of the method of Hecht and Potter (7). Liver was obtained from 3 month old rats (strain S) which were killed by bleeding under ether anesthesia. Slices of *ca* 500 μ thickness were cut with a Stadie-Riggs slicer and were incubated in 0.1% glucose-Krebs Ringer solution containing phosphate buffer. To liver slices of 500 mg wet weight in 3 ml of the medium, 0.5 μc/ml of orotic acid-6-C^{14} and 25–400 μg/ml of crude oncotrephin were added. Incubation mixtures were gently shaken in Warburg flasks for 4 hours at 37.5°C, the gas phase

being a mixture of 95% O_2 and 5% CO_2. In control experiment crude oncotrephin was not added.

After incubation the slices were taken out and a 20% homogenate was prepared from each in a glass homogenizer. From each homogenate, the RNA fraction was isolated by Schmidt and Thanhauser's method (8) and the radioactivity was counted in a gas flow counter.

Results

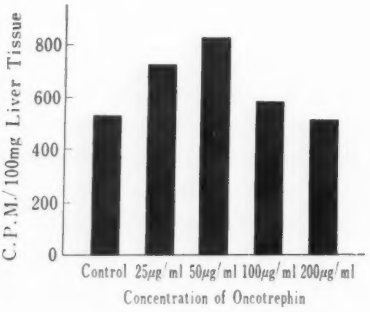


Fig. 1. Effect of Oncotrephin on Incorporation of Orotic Acid-6-C^{14} into RNA of Rat Liver Slices (Crude Oncotrephin Prepared from Human Hepatoma)

In comparison with the control an increase in incorporation of ^{14}C-orotic acid into RNA was constantly observed if the optimal amount of crude oncotrephin either from the human hepatoma or from the rat ascites hepatoma was added to the medium (Fig. 1). The optimal amount was estimated to be 50 μg/ml in both hepatomas. There was a maximum of 50% increase in incorporation.

Summary and Conclusion

Oncotrephin accelerates the incorporation of ^{14}C-orotic acid into RNA of liver slices *in vitro*.

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Vol. 52, No. 4 December 1961

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GANN

THE JAPANESE JOURNAL OF CANCER RESEARCH

Founded in 1907 by K. Yamagiwa

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Published by
THE JAPANESE FOUNDATION FOR CANCER RESEARCH
Nishi-Sugamo, Toshimaku, Tokyo, Japan

GANN, The Japanese Journal of Cancer Research, is intended for the publication of original articles in the field of cancer research. It is issued quarterly in March, June, September and December.

All communications concerning this journal, including manuscripts submitted for publication, should be addressed to Dr. Waro Nakahara, Editor of GANN, Japanese Foundation for Cancer Research, Nishi-Sugamo, Toshima-ku, Tokyo.

Subscription Price for foreign countries is $6.00 per year (per volume), post free. Foreign subscriptions are handled by the following authorized agents:

Charles E. Tuttle Co., 15, Edogawa-machi, Bunkyo-ku, Tokyo.

Nauka Ltd., 2-2, Kanda-Jinbocho, Chiyoda-ku, Tokyo.

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GANN, the Japanese Journal of Cancer Research
第52巻第4号
昭和36年12月25日 印刷
昭和36年12月31日 発行
編集兼発行代表者 中原和郎
東京都豊島区西巣鴨2丁目
印刷者 加藤保幸
東京都千代田区神田三崎町2ノ12
印刷所 株式会社 加藤文明社
東京都千代田区神田三崎町2ノ12
発行所 財団法人 癌研究会・日本癌学会
東京都豊島区西巣鴨2丁目 電話 東京(971)5186~8番
取扱所 東京都千代田区神田駿河台3ノ9
電話 東京(291)代表7121~7番 共立出版株式会社

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